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13. ABSTRACT *(Maximum 200 Words)* For drugs that interact with DNA, measures of DNA damage can assess the intracellular availability of active drug at a critical molecular target. Measurements of DNA damage should reflect the integrated effect of all resistance factors, including both recognized mechanisms and uncharacterized mechanisms. Thus, molecular measures of DNA damage could provide an important tool for elucidating the time course of complex changes in resistance factors. Motivated by a recent clinical trial that demonstrated better survival when the interval between induction chemotherapy and high dose therapy was prolonged, this project is using measures of DNA damage to determine whether induction chemotherapy causes transient changes in resistance. Findings indicate cyclophosphamide, cis-platin, and BCNU each produce DNA damage that can be measured in a dose dependent manner. Using a laser scanning cytometer, individual cells can be identified on slides. The characteristics of the individual cells (e.g., the presence of markers for normal or cancer cells) and cytoplasmic features (e.g. markers for apoptosis or enzymatic or other factors associated with resistance to chemotherapy) can be characterized by immunohistochemistry. Assays for DNA damage can be conducted in the same cells and associations between DNA damage and cytoplasmic features determined at the individual cell level.

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Introduction

The immediate goal of this work is to contribute to understanding of the unexpected schedule dependency observed between induction and high dose therapy in a clinical trial of high dose therapy with cyclophosphamide, BCNU, Cisplatin and marrow or autologous stem cell support (CPB-ACS) (1). That trial found that CPB-ACS was more successful when used at relapse (CR, Observation arm in Figure 1, overall survival 40%) than immediately after induction therapy (CR, Immediate Arm in Figure 1, overall survival 20%)(1). Understanding the basis for the schedule dependency of this treatment program would directly facilitate development of more effective scheduling for this and potentially other high dose chemotherapy programs. In a broader context, work done here to develop measurements of DNA damage and molecular dose for clinical oncology will provide a rapid way to measure the effects of different treatment programs. These measurements would provide an approach to develop optimal treatment protocols with many fewer patients on a more rapid time frame than could be achieved using clinical outcomes. This project will contribute to these goals by developing methods for measuring resistance to chemotherapy in clinical specimens and using the measurements to help design the optimal timing between courses of therapy for high dose chemotherapy.

Body of Report

A summary of our accomplishments with respect to the goals of the project in the Statement of Work are as follows:

Task: Obtain specimens of tumor, blood cells, and other tissues from high-dose chemotherapy studies for analysis.

This research project was motivated by a previous clinical trial that demonstrated better survival when a prolonged interval was used between induction chemotherapy and high dose chemotherapy (1). A total of 423 patients with biopsy proven metastatic breast cancer, which was hormone receptor negative or had progressed while receiving hormonal therapy, were entered into the study between 1988 and 1995 (1). No prior chemotherapy for metastases was permitted, although 50% of patients had received prior adjuvant chemotherapy. The randomized trial evaluated the use of induction therapy of andriamycin, 5-fluorouracil, and methotrexate (AFM) plus high-dose combination chemotherapy using the alkylating agents (cyclophosphamide, cisplatin, and carmustine (BCNU) (CPB)) (1). If a complete response (CR) was achieved to the induction therapy then the patients were randomized either to immediate consolidation with high-dose CPB treatment (1 week) or delayed treatment after the patient relapses (median 21 weeks after response evaluation) (Fig. 1a) (1). Bone marrow and/or mobilized peripheral blood progenitor cells (PBPC) were harvested from all patients at the time of response evaluation and were cryoperserved until completion of high-dose therapy. PBPCs were administered soon after chemotherapy was completed (1).

Patients received two to four cycles of AFM therapy until complete remission or best response, 98 patients achieved a complete response to the induction therapy and were randomized into the different groups. In designing the trial, it was believed that high-dose

chemotherapy should be more effective when the tumor burden was low. Therefore, the researchers expected to see a better long-term outcome for patients receiving high-dose consolidation immediately after induction therapy at the expense of increased immediate toxicity. However, this premise turned out to be incorrect: the survival rate of patients given treatment at the time of recurrence (delayed arm) was two-fold greater than patients on the immediate arm (Fig. 1b) (1). The median follow-up for both arms is in excess of 5 years. This difference has been durable for at least two to three years.

This research project was designed to understand the results of this randomized AFM trial. We developed and employed molecular methods to determine the levels of DNA damage caused by the CPB change when administered at different times after induction therapy. The hypothesis we hoped to demonstrate over the course of this research work is that before any drug treatments the patient would have very low resistance and a high amount of damage (Fig. 2). After the induction therapy, the resistance to the drug increased making treatment with high-dose chemotherapy not beneficial at this time period. Cellular resistance decreased and the DNA damage would increase after some time has passed or after the patient relapses. Therefore, several weeks after induction therapy would be the most beneficial time to give high-dose chemotherapy because it would cause the most damage to the DNA and kill the tumor cells.

This hypothesis reasons that better survival occurred when the interval between induction therapy and high-dose chemotherapy was prolonged because AFM induction therapy reversibly altered the expression and/or activity of a factor influencing sensitivity to the high dose alkylating agents (e.g. Glutathione S-Transferase, multi-drug resistance protein, DNA repair or other enzymes) (2, 3). Therefore, cellular resistance is increased after induction therapy, but decreases at the time of relapse. For a mechanism of resistance to be relevant, it would be

responsible for resistance to alkylating agents and it would have to be inducible by the induction therapy. It should remain at high levels for at least 1 to 2 months, possibly through continued stimulation, conceivably by immune or inflammatory responses following cell death and destruction. Finally, the system should return to pre-induction levels after a certain period of time.

The goal of this work is to prove the above stated hypothesis in patient samples and cell lines by monitoring DNA damage and cellular resistance after *in vitro* treatment with a high-dose chemotherapeutic drug. The samples used are bone marrow aspirates from breast cancer patients from a repository previously developed by our bone marrow transplant program at different time intervals after drug induction chemotherapy. These intervals include pre-induction, post-induction evaluation one and two, and after the patient relapses. Samples may or may not have micrometastasis to the bone marrow.

During the course of this project, however, new information concerning the clinical effectiveness of high dose chemotherapy impacted the project. From the mid-1980s to the mid-1990's, interest in the treatment of high-dose chemotherapy grew due to promising data in early clinical trials. Data from the initial Phase I trial of high-dose chemotherapy with CPB mentioned above demonstrated high-dose chemotherapy has an extremely high objective response rate (about 70%), including complete responses in 17 to 37 percent of patients (1). Phase II trials found that combining induction therapy with high dose therapy increased the complete response rate to 40 % of patients. Comparisons of the data with historical controls prompted high interest in this procedure among women with breast cancer both in and out of clinical trials (4). The therapy looked very promising as a standard of care for the treatment of primary breast cancer.

In 1999 and 2000, however, early results of randomized trials did not show an advantage for this approach over conventional therapy and the therapy became a controversial subject for patients and physicians. One of the reasons for this controversy was the negative media coverage surrounding and subsequent to the presentation of preliminary findings reported at the 1999 American Society of Clinical Oncology (ASCO) meeting. News anchors were presenting data before the official posting and before co-investigators saw the data. Within 24 hours of the ASCO posting of the abstracts, editorials presenting policy conclusions were published in the New York Times by individuals who never saw the data (5). This one-sided reporting has resulted in confusion and anxiety for patients with poor prognosis disease who are facing difficult decisions and for the doctors advising them (5).

Another controversy surrounding the therapy occurred later in 2000 and 2001, when the South African investigator Dr. Werner Bezwoda admitted to data falsification in a study of high-dose chemotherapy. The study had reported improved survival for patients in the high-dose treatment arm of a randomized comparison with conventional therapy. With the exclusion of that report, the available data indicated that a very large advantage for high dose chemotherapy as used in those studies was unlikely. The data could not exclude high dose therapy being somewhat more effective than standard therapy, or that it would be of great benefit with further modifications (4, 6, 7). This negative publicity dramatically reduced accrual to clinical trials, patient recruitment, and investigators interest in this type of therapy (8). For example, the clinical trial described in our initial proposal was designed to study 235 women in each of 3 arms for a total of 795 women. Between 1998 and 2000 less than 50 women entered the trial. While we planned including a small subset of the patients on this trial (ones on whom serial bone marrow specimens were taken for clinical purposes) for this study, low accrual made it infeasible

to complete the effort here with new specimens. We therefore relied on specimens collected for other purposes in our repository. Those specimens, however, had to be shared with other studies. That required our developing techniques that could meet study goals with reduced numbers of cells. Since the specimens were cryopreserved in liquid nitrogen and could not be refrozen without potential damage, we also had to conduct our analyses simultaneously with work planned for the specimens for other projects. As discussed below, simultaneous analysis actually provides large scientific advantages: it allows a cell-by-cell comparison of data produced for this project with other information that will be obtained from the samples. But this in turn greatly increased the technical complexity of the DNA damage analyses.

Therefore, we invested much effort in development of assays for DNA modifications that use a dramatically smaller amount of cellular material than was required for our initial pilot and developmental work. The amount of specimen needed for analyses of DNA damage is less than 2000 cells and 200 nanograms of DNA. These assays measure resistance as it occurs *in vivo* by exposing the cells *in vitro*. The technical advances that allow the use of this *in vivo/in vitro* approach allowed us to study samples from the same patient obtained at different time points. This resulted in our being able to analyze specimens from the same group of patients at each time interval required for the study, rather than obtain separate samples from patients in different chemotherapy arms. Thus the same patients could be used to study the effect of therapy by comparing pre-treatment samples with samples obtained soon after induction therapy and at the time of recurrence, similar to Arm A, B, and C in the clinical trial described above.

Finally, although designed around an observation and studies of high-dose chemotherapy, the data and methodological advances obtained from this project is equally applicable to

understanding resistance in conventional dose chemotherapy. We are confident it will provide useful contributions whichever treatment is the standard for care in the future.

We analyzed specimens of mononuclear cells isolated by ficoll gradients of bone marrow aspirates from patients prior to CPB therapy available from our repository. These specimens were cryopreserved using a step-down freezer and stored in the vapor phase of liquid nitrogen for less than 3 months. Specimens were rapidly thawed, washed, incubated with BCNU for two hours, and analyzed by the alkali SCGE assay. Damage was readily and quantitatively detected in these cells, indicating they will allow analyses of alterations in resistance after induction therapy.

During year 2 of the grant the limited availability of specimens prompted a reassessment of the potential role of other sources of archived samples that could be used. For example, the transplant program at Duke University (where the original trial cited in the proposal was performed and the Principle Investigator of this project holds an Adjunct appointment) had over 1800 specimens obtained during the course of the trials. Unfortunately the preservation methods used for these samples were not ideal for the standard SCGE assays. We have therefore expanded our emphasis on use of DNA damage assays that would be feasible with these specimens.

The timing of use of these samples had to be coordinated with other uses of the samples required our extending the period of the project through no cost extensions. Wherever possible, methods were developed using cell lines to preserve the patient samples. While as yet incomplete, we continue to plan to analyze approximately 80 specimens obtained prior to and soon after the completion of induction chemotherapy, and at relapse. Most of the patient

samples will be analyzed for all three time points and treated with a control and one or two doses of drug for each specimen.

The ability to use serial specimens from the same subjects allowed us to use existing specimens from studies of patients who underwent high-dose therapy. These specimens include between 20 and 30 suitably cryopreserved specimens for each of the time intervals (induction therapy, 8 weeks after therapy prior to induction therapy, and at the time of recurrence) required for our study. Use of these specimens and subjects also allows comparisons of DNA damage levels with the data being obtained through ongoing studies of these patients, including pharmacokinetics, cellular resistance factors, and tumor vascularity by other projects in our bone marrow transplantation program.

Tasks: Further develop assays for DNA adducts formed by the chemotherapeutic agents under study in order to optimize them for studying in vivo effects in patient specimens; development of in vitro approaches to studying adducts in short term primary culture.

During the first and second year of the project, we conducted studies of several methods for analyzing DNA damage chemically (SOP-1, A-F). We isolated DNA by several methods, obtained adduct standards and HPLC columns suitable for chemical analysis, and procured components of instrumentation needed for chemical assays. Feasible chemical analyses of the addition products have the advantage of specificity for the agent being administered. This is true in instances where larger samples of DNA are available and most desirable for discerning the effects of a single agent. The high doses used clinically, the fact that it is the third drug given in the combination under study, and availability of a highly sensitive HPLC based assay for BCNU adducts has led to us to emphasize the analysis of BCNU chemical addition products (Figure 3).

We used the analytic approach developed by Ye and Bordell (9)for these analyses. Methods and preliminary findings are presented in the Appendix (SOP-1). This work demonstrated that it is technically feasible, but requires substantially more sample than the single cell gel electrophoresis (SGE) and polymerase chain reaction (PCR) based approaches, and tends to be less reproducible than those approaches. The DNA isolation work (SOP-1b) indicated that we obtained about 1 ug of suitable DNA per ml of blood. While this will provide sufficient material for chemical analyses *in vivo*, where a single isolation of DNA will be used for several assays, the quantities required and difficulty isolating very small amounts of DNA will preclude conducting dose-response curves with replicate analyses *in vitro* using the chemical approach. These considerations prompted us not to favor continuing this analysis with the patient specimens, but focus on alternative techniques.

Progress in polymerase chain reaction (PCR)-based approaches to adduct analysis in the late 1990s provided a complementary approach to quantifying adducts (10, 11) (12, 13) (14). The methods rely on the observation that drug-DNA adducts block the progress of *taq* polymerase. Thus in the presence of DNA-adducts a repeated unidirectional PCR will accumulate multiple copies of truncated product. Alternately isolation of the specific fragments and ligation of sequence for a reverse PCR primer allows geometric amplification of product. These methods both allow adduct detection with very small quantities of DNA. Methods for this approach are presented in reports by Grimaldi and Hartley (10-13) and our SOP for initial steps in this work is presented in SOP-1d.

We conducted work toward making this assay approach higher throughput than that described in these reports. We used the primers measuring DNA damage in the Human N-ras, intron 1 sequence to demonstrate the measurement of PCR product accumulation by monitoring fluorescent accumulation using the using real time polymerase chain reaction capabilities of a I-Cycler, a thermocycler with an fluorescent optical monitoring head. Analysis showed no accumulation of product until cycle 18 and then exponential accumulation of product between cycle 23 and 28. This work suggested that detection of changes in PCR could be monitored by real time fluorescent monitoring. In addition to allowing many more reactions to be run for the same effort, this approach eliminated the need to use multiple specimens in order to identify when the sample is undergoing expontential PCR required for these assays. This in turn allowed assessment with much less sample than would be required by gel-based approaches. While the assays proved feasible, the amount of starting material exceeded that available from many of the patient samples. This prompted concentration on the comet assays for subsequent work.

Studies performed subsequently emphasized the use of the Single Cell Gel Electrophoresis (SCGE) Assay, otherwise know as the Comet Assay, to monitor levels of DNA adducts. This assay is highly sensitive and can be performed on small numbers of cryopreserved cells. For the *in vitro* studies to be used for this project, this technology allows analysis at multiple dose levels and replicate assays with small numbers of cells that would not be feasible with other approaches. This project used the Comet Assay to detect the DNA damage in the metastatic tumor cells, normal bone marrow cells, as well as breast cancer cell lines such as MCF-7. In outline form, the protocol for the experiment consists of treating the cells with various concentrations (0, 100, 300, 1000 μM) of the high dose chemotherapy drug for a standardized two hour incubation at 37 C and looking at the differences in the amount of DNA damage at the different time intervals. For the samples with metastasis, magnetic beads were used to separate out the tumor cells from the bone marrow. Cells are stained by immunocytochemistry, the cells mixed with agarose, and layered on slides for electrophoresis. After electrophoresis an Image Analysis System or Laser Scanning Cytometer (LSC) was used to measure the DNA damage and cellular resistance of the cells by examining the patterns of fluorescence of the cells, as described below.

The Comet Assay provides an attractive approach for measuring DNA damage in clinical samples. It involves isolation of cells, embedding cells in agarose and adding them to a slide, lysing cells to remove membrane and proteins, denaturing DNA, electrophoresing cell remnants (nucleoids), neutralizing, drying, and staining cells with DNA fluorescent dye (Fig 4) (2, 3). The stained nucleoids are examined using a fluorescent microscope and an image analysis system or Laser Scanning Cytometer. The structure of the nucleoid consists of DNA organized as loops, which retains the supercoils that were formerly contained in the nuclesomes (15). The electric

current pulls the charged DNA from the nucleus such that the relaxed and broken DNA fragments migrates further. Nucleoids with intact DNA remain circular after electrophoresis. Cells with DNA breakage reveal a brightly fluorescent circular head and a tail of damaged DNA that has electrophoresed faster than the intact DNA to look like a comet, hence the name 'Comet Assay'. We also choose to expose cells *in vitro*, because it allows measuring the effects of each agent independently. It also facilities carefully controlling analyses studying the same patient at several time points. Using this design each patient serves as his own control for timecourse effects, maximizing statistical power.

For most analyses the SCGE assay was performed under alkaline conditions using an adaptation of the method of Singh et al (16). To summarize the initial method we used, 100 ul of treated cells were suspended in 200 ul of 1% low melting point agarose in PBS at 37°C. 100 ul of the cell suspension was pipetted onto a frosted glass microscope slide pre-coated with 1% normal melting point agarose and covered with a coverglass. After gelling for 5 min at 0°C, the coverglass was gently removed and a third layer of 100 ul LMP agarose was added and covered again with a coverglass. After gelling for 5 min the second time, the slide was put in a tank filled with the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, NaOH to pH 10.0, 10 gms Sodium Dodecyl Sulfate (SDS) electrophoresis grade, 1% Triton X-100, and 10% DMSO) at 4°C for at least 4 hours. The slides were next placed in a horizontal electrophoresis tank containing 300 mM NaOH and 1 mM EDTA for 20 min at 4°C to allow for unwinding of the DNA prior to the electrophoresis at 25 V (1 V / cm, 300 mA) for 30 min. The slides were then washed twice for 5 mins each with cold neutralization buffer, 0.4 M Tris-HCl, pH 7.5. Finally the slides were immersed in ethyl alcohol for 5 min before staining with SYBR Green 1. For these procedures plastics were from Corning (NY,USA), agarose and Tris were from Fisher

Scientific, Chicago, (IL, USA), and the SDS was from Bio-Rad Laboratories (CA, USA). The trypsin, salts, and buffers used in the lysis solution and the electrophoresis buffer were from Sigma, St. Louis (MO, USA). The Standard Operating Procedure (-) is in the Appendices section of this report (SOP-2).

The Comet Assay evolved from previous methodologies used to assess DNA damage. In 1978, Ryberg and Johanson were first to directly quantitate DNA damage in individual cells based on differential lysis of irradiated cells in alkali, later they used this technique for flow cytometry (17). In 1984, Östling and Johanson wanted to improve the sensitivity of the technique, so they developed a microelectrophoretic technique for the direct visualization of DNA damage in individual cells (18). They used a neutral method that appeared to be sensitive to the loss of DNA supercoiling caused by single-strand breaks. Electrophoresis acted to pull negatively charged damaged DNA away from the nucleoid towards the anode. Cells with an increased frequency of DNA double-strand breaks displayed increased migration of DNA towards the anode (18). They observed that the extent of DNA liberated from the head of the comet during electrophoresis was a function of the dose of irradiation. Their paper presented an evaluation of DNA damage in cells recovered from biopsy samples of patients receiving radiation therapy. This paper led to similar observations in other laboratories that may ultimately allow prediction of tumor responses to radio- and chemotherapy regimens.

During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring, DNA repair processes, and genetic toxicology (19). The Comet Assay has numerous advantages over other methods for measuring DNA damage. These advantages are it is highly sensitive to detecting low levels of DNA damage, only a small number of cells are required, almost any eukaryotic cell

can be assayed. It allows for rapid cost effective experiments and the cells can be cryoperserved prior to analysis. Slides prepared for the assay can be dried, stored, or restained for later analysis. There are several comprehensive reviews covering the history, methodology, and applications of the Comet Assay (19-23).

The comets follow similar DNA migration patterns that distinguish the amount of damage to the cell. The size of the DNA, number of broken ends, and the dosage of drug used control the level of DNA migration. At low levels of damage, stretching of attached strands of DNA, rather than migration of individual pieces occurs. Increasing the dosage will increase the tail of the comet and the number of strand breaks. Thus, causing the DNA pieces to migrate freely into the tail of the comet. At an extreme amount of damage (apoptotic cell), the head and tail are well separated. The intensity of the fluorescence in the tail relative to the head provides information about the number of strand breaks. Figure 5 shows the migration pattern of MCF-7 cell comets at different drug concentrations 0 μM (a), 100 μM (b), and 300 μM (c) of BCNU from one of our Comet Assay experiments. The figure shows a large increase in the size of the comets for both of the two higher doses of drug, while the control shows barely any damage. However, in the highest dose there is a larger space between the head and the tail of the comet, indicating apoptosis.

Currently, there are different versions of the Comet Assay used in laboratories all over the world. The most popular Comet Assay technique and the one we used in the beginning of this project is the Single Cell Gel Electrophoresis (SCGE) technique. SCGE was introduced by Singh and colleagues in 1988 (16) and uses alkaline electrophoresis (pH>13) to analyze DNA damage after treatment with X-rays or H_2O_2. Alkali caused denaturation of the duplex DNA, and allowed the individual strands to separate and migrate independently, making it possible to detect single-

strand breaks (SSB), SSB associated with incomplete excision repair sites, and alkali labile sites (ALS) (15). The separation of DNA strands is facilitated by the extent or rate of separation and is the index of DNA breakage. The comet tail is made up of relaxed loops, and the number of loops in the tail indicates the number of DNA single-strand breaks and double-strand breaks (DSB) (15). Because almost all genotoxic agents induce orders of magnitude more SSB and/or ALS than DSB, this version appears to be at least one- or two-orders of magnitude more sensitive for identifying genotoxic agents (24). In 1990, Olive et al. (25) introduced the name 'Comet Assay' and developed a version of the neutral technique introduced by Ostling and Johanson, which involved lysis in alkali treatment followed by electrophoresis at either neutral conditions to reveal double strand breaks (25, 26) or mild alkaline conditions (25, 26) to detect single strand breaks.

We assayed specimens available from a repository in which specimens were obtained, processed, and stored at -80 deg C for over 2 years. These specimens had a very high background of damage in the pretreatment samples, levels that precluded their use for studying chemotherapy induced damage (data not shown). A variant of the SCGE assay, however, has allowed us to use the several year old cells to study both *in vivo* effects and *in vitro* changes in sensitivity to damage. This assay uses the Olive method neutral conditions instead of alkali for lysis and electrophoresis: lysis is done in 30 mM EDTA, 0.5% Sodium Dodecyl Sulfate at pH 8.3, and electrophoresis is conducted in TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA). We have determined that this assay is extremely sensitive to crosslinking. As in the standard assay, cross-linkers contract the cells, resulting in smaller tail moments. This occurred in 8 of 10 patients following CPB therapy. It also occurred in all instances following *in vitro* exposure to CisPlatin.

The need to analyze small numbers of cells led to further assay development. Bacso et al.,(27) describes further developmental work with the SCGE assay, which allowed us to use the same cells that are analyzed for cellular damage endpoints for SCGE assays of DNA damage. For these assays cells were stained with annexin V (a marker for apoptosis) and/or propidium iodide (a marker for cell viability). Three hundred ul of low melting point agarose was added to each 100 ul aliquot of cells, and 80 ul layered on the surface of slides previously prepared with a base level of agarose. After solidification, an additional agarose layer was added. The slides were read using a laser scanning cytometer (LSC) using a 488-nm argon-ion laser and collecting green fluorescence and red fluorescence emissions for measurement of the cellular endpoints (described in the next section of the report). Cells could be scanned at a rate of approximately 300 per minute. After these readings the coverslips were removed and the slides were immersed in the lysis buffer described for the SCGE assay. The DNA was stained with SYBR Green, neutralized, and fixed as described above. The slides are then returned to the LSC and the same cells analyzed for fluorescence relocated by the LSC. These cells are then analyzed by the SCGE assay by transferring the video signal from the LSC to the Komet image analysis system (described in the next section). This dual capability will both increase the cell numbers available for DNA damage assays by allowing the use of cells from fluorescence assays for DNA damage assays and it will allow direct comparisons of fluorescence endpoints being conducted under other studies with the DNA damage studies being conducted here.

For the last two years of this grant, our developmental work improved the assays used to study induced resistance and DNA damage. Using the original protocol in conjunction with the LSC scanning of individual cells assays were hampered by problems with cell yield on the slide, distribution of the cells on the slides, and a long scanning time for the analysis of the slides. To

maximize the cell yield we decreased the volume of solution that the cells are suspended in before adding the agarose to the cells. This allowed more cells to be added to the slide because there was a higher concentration of cells in the cell and agarose mixture. The problems with cell distribution and scanning time of the slide were corrected by keeping the cells within a designated area on the slide. This was done by adding half of the amount of agarose and cell mixture onto the middle of the slide. Then a 22 mm coverslip was added over the mixture and the second layer of just agarose was added the same as before. Originally, to find a small number of separated tumor cells we would have to scan the whole slide and it would take about an hour/slide. This was not feasible for experiments consisting of multiple slides (as many as twelve samples per specimen) to be scanned all in one day. By keeping the cells in the middle of the slide scanning time was reduced by around 40 minutes and the cells were easily detected by the Laser Scanning Cytometer.

After revising the SCGE method, we found that slides designed for simultaneous processing of large numbers of samples for the Comet Assay had recently been developed by a small company Trevigen, Inc. Trevigen's CometAssay ™ uses a proprietary CometSlide ™ that is specially treated to promote adherence of low melting point agarose. This method provides a simplified and more effective method for evaluating DNA damage in cells than the original SCGE sandwich method. The use of CometSlides ™ shortens assay time and allows for a rapid and reliable analysis of large numbers of samples. The company offers versions with 2 wells and 20 wells. Trevigen also makes a Comet Assay Kit ™ which includes Comet LMAgarose, already prepared Lysis solution, CometSlides ™, 200 mM EDTA, pH 10, and SYBR^{R} Green Concentrate. These multi well slides allow analysis of samples from multiple time periods and multiple doses levels of exposure in a single slide. Compared with having each time period and

dose on a separate slide, having all specimens on the same slide allowed tight experimental control over electrophoresis and other processing steps. It allows rapid preparation and analysis of all of the patient samples. The SOP for this new procedure is in the appendices section of this report (SOP-3).

We conducted many experiments using the MCF-7 WT or adriamycin resistant (Ad-R) breast cancer cell lines, provided by Dr. Gerard Batist, at Lady Davis Hospital in Montreal, Canada to model the tumor cells found in the metastatic patient samples. The cell lines were used to streamline methods while conserving patient samples. The cells were cryopreserved in liquid nitrogen. The media that we used was EF (1:1 mixture of E-Media and F-Media), Fetal Bovine Serum (FBS), 5% FBS/EF, and Trypsin (the SOPs for the EF media preparation (SOP-4) and maintaining these cell lines (SOP-5) are attached in the appendices section of this report). The FBS and Trypsin are from Gibco Cell Culture, Invitrogen Corporation (CA, USA).

Summarizing procedures used for cell line maintenance, before working with cell lines to warm up all of the media in a 37 C in ¾ full water bath. To start a new cell line remove cryogenic vial from liquid nitrogen and place vial in water bath and allow it to thaw quickly. As soon as it is thawed, pour into a 15-mL centrifuge tube, slowly dilute with cold 5%FBS/EF, and add media dropwise to the thawed solution. Next, spin down the tubes at 2000 rpm for 5 minutes, then pour away the supernatant and flick cells with your finger. Add 1-2 ml EF to the pellet and mix up cell suspension with pipetting and count the cells on a hemocytometer at a 1:10 dilution with Trypan Blue. Trypan Blue is a cellular dye that helps to distinguish between the living and dead cells. Trypan Blue is excluded from the membrane of viable cells, whereas the nuclei of damaged or dead cells take up the stain.

To passage or to prepare the cells for an experiment, pour away or vacuum away old media from the flask. Rinse the flask with 1 ml of Trypsin and pour or vacuum out the trypsin. Next, add the last 1 ml trypsin and incubate at 37 C for 7-10 minutes. After incubation, add 4 ml EF (no FBS), wash bottom of flask, and pipette up and down until you get an even cell suspension. Transfer into centrifuge tube (15-ml) containing 1 ml FBS and centrifuge cells at 2000 RPM for 5 minutes and then count the cells as mentioned above. We passaged the cells about every other day, making two new flasks/passage and tried to keep the cell line going as long as possible. We found that the number of cells will double after about two days and a flask will be completely full after about 5 days. If not passaging the cells, it is necessary to change the media every other day to keep the cells alive. When removing cells for comet analyses use Cell Dissociation Media to avoid effects trypsin may have on baseline levels of coments.

The cell lines were used to model the effects high-dose chemotherapy has on the metastatic tumor cells in the patient samples and for comparison with normal bone marrow cells. The goal of antineoplastic goal is selective eradication of malignant cells, while leaving normal host cells viable (28). Unfortunately, the difference between the amount of drug needed to induce successful tumoricidal action and the amount needed to produce toxicity in the host is small (29). Therefore, it is necessary to determine the appropriate amount of damage to the tumor cells, while sparing the normal cells. The Comet Assay is useful for doing this because it evaluates DNA damage as a biomarker of exposure to a drug and facilitates the detection of SSB, ALS, DSB, incomplete excision repair sites, and interstrand crosslinks (28). It also enables the study of different repair pathways, such as base and nucleotide excision repair induced by the drugs, cell death and apoptosis.

We conducted a series of *in vitro* experiments using the cell lines to establish dose-response relationships for DNA breakage induced by each of the individual agents used for CBP. Each agent induced DNA breakage at doses comparable to estimated AUCs that would be achievable in vivo with CPB therapy. Qualitatively, at low doses the pattern of breakage was grossly similar for each agent. At high doses the pattern of damage differed for each agent. These patterns were consistent for most of the cells assayed. The patterns were also similar to those previously described in the literature for their DNA damaging mechanisms (30-32). These patterns were presented in Everson *et al.*, 2000, (2) cited under Reportable Outcomes and Fig 6 in the appendices section.

In these studies, as illustrated in the Fig 6., damage caused by high doses of:

- Cisplatin contracted the cells, consistent with its ability to cause DNA crosslinks and patterns of Comet damage previously seen with crosslinking agents.
- BCNU caused a pattern suggesting diffusion of relatively constant molecular weight fragments prior to electrophoresis and displacement of those fragments during electrophoresis, a pattern shown to indicate apoptosis in a recent series of studies.
- 4-hydroxy cyclophosphamide (4-HC) caused broad diffusion of the DNA, a pattern previously linked with necrosis.

We used the KOMET Image analysis system described below to qualitatively measure the comets. The levels of DNA damage as measured by the Olive tail moment (product of DNA fluorescence in the tail and tail length) increased with increasing concentrations of each drug. Each agent caused damage in a dose-dependent manner in both MCF7 cells and primary lymphocyte cultures, with flattening at very high doses of BCNU and 4OH-CP. Dose dependent

measurements of damage can be made for a broad dose range, avoiding levels associated with flattening or declining at high doses due to cell breakage.(2).

Cellular Resistance and Glutathione S-Tranferase MCF-7 Cell Line Experiments

We used the cell lines to develop new methods to study the multiple molecular mechanisms that are potentially incriminated in tumor chemoresistance (33), including the Glutathione redox system. Glutathione is a tripeptide composed of glutamate, cysteine and glycine. GSH has numerous important functions within cells: it serves as a reductant, is part of the peptidoleukotrienes, and aids in amino acid transport across the cell membrane. In addition, GSH is a cofactor for some enzymatic reactions, rearranges protein disulfide bonds, and lastly conjugates to drugs to make them more water-soluble. This system is known to be inducible, particularly in cells undergoing oxidative stress and it plays a central role in protection of cells from oxidant injury (34). Glutathione exists in the reduced form (GSH), and maintains a balance with its oxidized form (GSSG), a disulfide.

Glutathione participates directly in the destruction of the normal oxidative by-products of cellular metabolism, including the reactive oxygen compounds free radicals or oxiradicals. It is involved directly in the detoxification of foreign compounds by reducing toxic substances before they can damage other molecules or important parts of the cell. When these toxins combine with glutathione, they form a water-soluble compound that can be excreted once and for all by the kidneys. The highest concentration of glutathione is found in the liver which is the principal organ involved in the detoxification and elimination of toxic materials.

Drug resistance represents a major obstacle to successful cancer chemotherapy. Cellular resistance is dependent on the redox system involving glutathione. The enzymes responsible for catalyzing the conjugation of electrophilic compounds to glutathione are the Glutathione S-

Transferases (GSTs) (35). The GSTs are an enzyme family consisting of four cytosolic isoforms (α, μ, π, and θ) (36). Expression of GST-μ enzyme has been shown to detoxify BCNU (37) and the GST-α isozyme is involved in conjugation of glutathione to cyclophosphamide (38). It has been demonstrated that there is a correlation between drug resistance and expression of GST-π in primary tumors in patient samples and various resistant human cancer cell lines (39-42). The pi isozyme has been proposed as a biomarker in predicting local breast cancer recurrence (43).

We examined the expression of GSTs in breast cancer cell lines to better understand the effect of cytotoxic drug treatments on cellular resistance. We used immunofluorescence in solution and the Laser Scanning Cytometer to measure the three different isozymes of the Glutathione S- Transferase family. GST isotypes are stained with rabbit antihuman antibodies specific for each enzyme; lastly, a secondary goat anti-rabbit antibody (Molecular Probes) is incubated with the cells in solution. Fig. 7 shows MCF-7 cells that were treated with 300 μM BCNU, stained with GST-π and 488 Goat Anti-rabbit antibodies, and scanned on the LSC before the Comet Assay (Fig. 7a). Next, the Comet Assay was conducted on the same slide and stained with the DNA stain SYBR Green I (Fig. 7b).

To find the GST that best correlates with resistance to one of the high-dose chemotherapy drugs, BCNU, we tested three isozymes (π, μ, and α) with treated MCF-7 cell lines. Two slides were made up for each different enzyme: 200,000 MCF-7 cells with 300 μM BCNU and 0 μM BCNU for the control. The next step was to run the Comet Assay on these slides to observe the DNA damage. During this experiment, we discovered that the slides with cells incubated with GST-π and α demonstrated a higher fluorescence during laser scanning cytometry, as well as bigger and more easily seen comets. The slides with GST-μ were very difficult to observe and

not a lot of damage was seen (Fig. 8). These results correlate to the above-mentioned study that demonstrated BCNU is deactivated by GST-μ (37) and GST-π and α are activated in breast cancer cell lines (39-42), such as the MCF-7 cell line we used.

However, we also observed high levels of damaged cells on the control slides, which was unexpected. To test if the staining procedure was causing damage to control cells, we ran a parallel experiment treating 200,000 cells with four different concentrations of BCNU (1000, 300, 100, and 0 μM), skipped the staining steps, and advanced onto the Comet Assay. Without the GST staining procedure, a larger dose response and a higher percentage of damaged cells were observed. This proved that the staining procedure was causing added damage to the cells.

An important component of the antibody solution is the metal cadmium. It is used to protect the cells from damage during the manipulation of the cells from the pipetting, centrifuging, and staining. The high levels of baseline damage suggested that that the cadmium was not working effectively. Our standard procedure was to use cadmium in only the first wash. The addition of cadmium to all washes decreased the percentage of damaged cells in the GST treated slides was much lower, indicating that cadmium was required in all solutions.

We then used these procedures with the Trevigen CometSlides™ described above. The method for these slides is similar to the original Comet Assay and includes suspending cells in agarose, adhering them to the slide by adding one layer of the cells and agarose suspension, allowing the cells and agarose to adhere to the slide, lysing, electrophoresis with an alkaline solution, and staining with a DNA stain (SYBR Green or To-Pro 3), and scanning on the LSC. . There is only one layer of cells and agarose, making the method faster and cleaner. The Trevigen slides gave cleaner preparations, improving scanning and contouring when using the

LSC for the Comet Assay. Also, they allow a set of assays to be scored on the same slide and minimize LSC instrument time required to complete the scanning

Unfortunately several technical problems slowed progress with these slides. A major problem was that sometimes the cell layer did not adhere to the slides, such that one layer was removed during one of the steps of the Comet Assay. This problem was eliminated by increasing the gelling time for the cells to stick to the slide before being put into the Lysis solution. We also had to carefully adjust electrophoresis times to provide optimal levels of separation.

Several experimans compared the original Comet Assay slides with the new Trevigen reagents and slides. We used 10,000 MCF-7 Adr-R cells for the original Comet Assay and 1,000 cells for the Trevigen method. For each method, each cell sample was treated with one of three drug doses or a DMSO control (0, 100, 300, and 1000 uM). There were good controls for each of the methods. Both of the methods were successful but the original assay gave higher proportions of damaged cells (Fig. 9). This might be due to the higher cell count for the original method. There was one sample in this experiment that did not contain comets, perhaps due to a problem with electrophoresis (300 uM BCNU sample with the Trevigen slides). If this sample is disregarded then a good dose response can be observed (Fig. 10).

Top optimize DNA stains, we compared results with SYBR Green (Molecular Probes) and To-Pro 3 (Molecular Probes) after the Comet Assay. SYBR Green I nucleic acid gel stain is an exceptionally sensitive nucleic acid gel stain that has bright fluorescence when bound to dsDNA and low background in gels, making it ideal for detecting dsDNA in gels using laser scanners or standard UV transilluminators. When excited (425 - 500 nm) the DNA-bound SYBR Green emits green light using the Argon Laser on the LSC. The principle of the To-Pro 3

staining is the intercalating of the component in the DNA of the cells, this fluorophore is a cyanine nucleic acid stain that is impermeable to living cells. It is almost nonfluorescent unless bound to DNA or RNA. The binding affinity to double-stranded DNA is very high. The fluorophore, which has a peak absorbance at 642 nm and emission at 661 nm, is best excited by an HeNe laser on the LSC (633 nm). Figure 11 shows comets stained with both DNA dyes. We concluded that SYBR Green is the better stain for the comet assay and have used it for the rest of the experiments. The main reason is because the comets were easily detected and contoured by the LSC without a lot of background fluorescence. This is better for the scanning of the slides and also for visualizing the comets on the microscope. For the To-Pro 3 staining, it was difficult to focus on and observe the comets under the microscope. Therefore, it was difficult for the LSC to count these comets as cells on the slide. A high level of background fluorescence also does not make To-Pro 3 the stain of choice for the Comet Assay. The To-Pro-3 stain faded a lot faster than the SYBR Green, making it difficult to rescan and take pictures of these cells. With the SYBR Green staining, we were able to get strong images of the comets, as well as easily relocate the cells after the scan.

Tasks: Application of standardized assays for in vivo and in vitro assays for DNA adducts to determining whether the clinical effects of schedule differences could be mediated by intensity of molecular agents on DNA; analyze the data to determine the duration of the effect of AT on levels of adducts formed by CPB and association between adducts levels and other endpoints; use the adduct dosimetry data to develop approaches to the selection of induction regimes and optimizing the interval between induction and high dose therapy to be tested in clinical trials.

Standardized Assays for Clinical Studies.

Separation of tumor and normal cells by density Separation We investigated approaches to separating normal and tumor cells relying on cell density. Bone marrow specimens were mixed with a small number of MDA breast cancer cells and separated using the OncoQuick^R Kit (Greiner Bio-One). The kit was designed for the enrichment of disseminated circulation tumor cells from whole blood. The entire enrichment procedure can be completed within 45 minutes yielding not more that approximately 10^4 total mononuclear cells and a total enrichment factor of up to 6 log units (OncoQuick^R Manual). The method consists of a 50 mL polypropylene centrifugation tube that is separated by a porous barrier. The lower compartment contains the blue colored separation medium. The upper compartment accommodates up to 25 ml of the blood specimen to be investigated. During a 20 minutes centrifugation step the cells will be separated according to their different buoyant densities. The denser blood components such as erythrocytes and leucocytes migrate through the porous barrier into the lower compartment, thereby forcing the separation medium into the upper compartment. The less dense cell fraction, including the circulating tumor cells, will be enriched at the interphase layer formed between the plasma and the separation medium in the upper compartment. Figure 12 shows how centrifugation separates out the tumor cells. After a harvesting and washing step the tumor cells

are ready for further processing. The SOP used for these separation experiments is SOP-6 in the Appendix.

The bone marrow sample cells and the MDA cells were counted and a mix of 250,000 MDA cells/ml and 5 million bone marrow cells/ml (1:20) was prepared for the separation experiment. After centrifugation, the layers were separated successfully, but we didn't see any pellet at the bottom of the tube. To determine the proportion of tumor and normal cells, we made cytospin slides with these separated cells and used immunohistochemistry to stain the cells for cytokeratin (discussed below). Tumor cells would take up the cytokeratin and show a red stain and bone marrow cells would be blue under the microscope. After staining we observed many large red cells and few blue cells. For the red cells (Cytokeratin positive) there were an average of 510 cells and for the blue cells an average of 201 cells. Thus after separation the ratio is 1 bone marrow cell for every 2.5 tumor cells. So purification of tumor cells was achieved. But the cells had to be further purified by multiple centrifugations in media, such that recover of cells was not optimal from bone marrow specimens. Therefore we continued to evaluate other methods that use a immunologic rather than density for cell separation.

Positive Immunomagnetic Cell Isolation. Breast cancer cells can be identified in and separated from bone marrows by immunocytochemical (ICC) methods. Immunocytochemistry uses an antibody against a cell protein or antigen, typically on the cell surface. The most widely used marker for metastatic breast cancer cells in bone marrow is cytokeratin because breast cancer cells but no normal marrow components are cytokeratin positive (44, 45). Cytokeratins are represented in epithelial tissues by at least 20 different polypeptides. Cytokeratin 18 is expressed in columnar epithelial cells from digestive, respiratory, urogenital tract, endocrine and exocrine tissues and mesothelial cells (46). The European International Society of

Hematotherapy and Graft Engineering group for standardization of tumor cell detection introduced guidelines for analysis and evaluation of isolated tumor cells by immunocytochemistry (45). We used this immunological property of breast cancer cells to separate them from normal cells in bone marrow. The method we used was a positive Immunomagnetic Cell Isolation Method with the CELLectionTM Epithelial Enrich Positive Isolation System from DynalBiotech. Immunomagnetic cell separation has been shown to be a reliable technique for enriching contaminating tumor cells in blood and bone marrow aspirates. The micrometastatic cells are separated from the bone marrow sample using a selective enrichment method that selects the tumor cells by incubation with superparamagnetic, polystyrene beads (4.5 μM diameter) coated with rat monoclonal antibody (Mab) against an anti-EpCAM (epithelial cell adhesion molecule) mouse IgG1. The rat Mab is biotinylated and attached to the Dynabeads via streptavidin and a DNA linker (47)(Fig. 13). During a short incubation period on a mixing device, the epithelial cells bind to the beads, and subsequently, the rosetted cells can be isolated and washed using a magnet (Dynal Magnetic Particle Concentrator (Dynal MPC)). The beads are attracted to the magnet and the remaining cells not binding to the beads can be removed from the tube. This method allows several logs of enrichment of the cell population of interest, permitting screening of a large number of live cells. This results in highly sensitive detection and facilitates further characterization of the selected micrometastatic cells.

Some of our first experiments consisted of spiking MCF-7 tumor cells into normal bone marrow samples at various cell numbers and then separating them out using the magnetic beads. There are two different protocols for using the Dynal Magnetic Beads including keeping the beads attached and also using DNase to remove the beads from the separated tumor cells (SOP-7 in Appendices). In order to examine if the DNase treatment causes added damage to the cells,

we treated MCF-7 Adr-R cells with DNase and performed the comet assay. This experiment demonstrated that the DNase did not cause added damage to the cells. The next step was to find the volume of beads used to optimize the number of cells added to the slide. For the first experiment, 20 μl of beads were used and it was observed that a large number of beads interferes with the analysis and scanning procedure, actually covering some cells to an extent that they cannot be seen. Using a smaller number of beads allows the observer to see a small number of beads rosetted to the cells under the microscope, identifying it as a tumor cell. After finding the right bead volume we wanted to test if removing the beads had as successful a recovery as keeping them attached. There was a better recovery of tumor cells with the beads attached (159 cells) than with beads removed (46 cells), so we adopted the procedure that left the beads in place.

This cellular enrichment and staining method was worked successfully with when relatively large numbers of tumors cells were being added to each bone marrow sample. Each of the experiments had three different set of slides treated with the different drug doses. The different slides contained MCF-7 and bone marrow cell mixtures (1:400), 10,000 MCF-7 WT cells only, and 10,000 BM cells only stained with the Cytokeratin and GST antibody mentioned above. After the Comet Assay, the slides were scanned to measure the % of DNA damage in the cells on the WinCyte Software. Figure 15 shows the dose response for a cell separation experiment.

When the tumor cell numbers were reduced to around (1000-100 MCF-7 cells) we were initially not getting good recovery of the cells using the beads. The multiple steps in the protocol appeared to lead to a large amount of cell loss. The cell loss could have been attributable to the combined effects of several factors in the assay including: the number of mononuclear cells

analyzed, the expression of antigen used for the detection of tumor cells, the specificity of the monoclonal antibody used, the multiple centrifugation steps needed to prepare the cells for the immunocytochemistry and mixing the cells with agarose for the comet assay. It was very important to mix the cells and agarose thoroughly to get an even distribution of cells on the slide for the Comet Assay.

Because of their advantages discussed above, we used the new multi-well Trevigen CometSlides^{TM}. However, we experienced a series of problems with these slides. Initially we saw great variability in the comets among different wells on a slide, typically with some wells showing adequate numbers of sharp images comets but for others few cells remained on the slides. The problems appeared to be related to the ratio of the volume of media used to suspend the cells and the volume of agarose. Figure 16 shows dose response curves for three separate experiments using the Multi-well slides. This graph demonstrates how some of the experiments showed better dose response curves than others. The optimal volumes for media and agarose determined in these experiments are presented in the SOP-3 in the Appendices.

Identification and Differential Scoring of cell types by immunocytochemistry In addition to separating cells by ICC methods, we used them to distinguish normal marrow and breast cancer tumor cells so that the two cell types could be scored simultaneously in our specimens. ICC methods for identifying breast cancer cells are exquisitely sensitive, and can detect as few as one to two tumor cells in 1*10^6 bone marrow mononuclear cells (48). For identification of cytokeratin positive cells, we stained the tumor cells with an antibody that recognized human cytokeratin 18 (48 kDa protein) (46) in order to observe and measure the fluorescence on the Laser Scanning Cytometer (Fig. 17). The antibody used is a purified mouse anti-human cytokeratin 18 monoclonal antibody (BD PharMingen 550508). To see the cells under a

fluorescent microscope it is necessary to label the monoclonal antibody with a fluorescence marker. The antibody labeling kit I used was Alexa Fluor 647 Monoclonal Antibody Labeling Kit (Molecular probes A-20186). Conjugates produced with the bright and photostable Alexa Fluor 647 dye have absorption and fluorescence maxima of approximately 650 nm and 668 nm, respectively.

Image Analysis Systems Many investigators have used some form of image analysis for the Comet Assay: developing their own systems, using the NIH image software available on the Internet, or purchasing commercial software. Image analysis has become essential for objective measurement of low-dose effects, or for distinguishing small differences among subpopulations of cells (21). Direct microscopic examination (15) remains useful for observing larger differences (e.g. screening drugs or measuring the percentage of apoptotic cells.). There are many different parameters that can be measured using image analysis to analyze comet assays. Tail length, while relatively simple to measure, varies with electrophoresis conditions and equipment, specific fluorescent DNA stains, camera sensitivity, DNA content and image analysis. The percentage of DNA in the tail tends to be a more stable and sensitive measurement. Tail moment, originally defined as the average distance migrated by the DNA multiplied by the fraction of DNA in the comet tail, is also a relatively stable and sensitive measurement.

KOMET Assay Software Our first quantitative measurement of the levels of DNA damage was determined by staining with SYBR Green and then examining the cells using a Zeiss epifluoresence microscope with 40 x Plan-neoflur objective and a 50 w mercury power source and appropriate filter. The microscope was attached to an imaging system consisting of a intensified CCD camera and a IBM compatible PC computer with a KOMET Assay Software

(Kinetic Imaging, LTD). From each slide typically 50 images were analyzed sequentially; only overlapping cells were omitted from analysis. The image analysis algorithm used first applied an edge filter to define the limits of the comet, then subtracted the background (defined as the image intensity at the edge of the comet), and subsequently formed head and tail distributions for analysis. The Olive Tail moment, defined as the product of the amount of DNA in the tail multiplied by the tail length (distance between edge of comet head and end of tail), was used as a measure of the amount of damage in individual cell (49) (50).

Laser Scanning Cytometry. In order to increase the sensitivity, speed, and objectivity of the analysis, we have developed measurements based on image analysis using a Laser Scanning Cytometery (LSC). This advanced image analysis system can determine size, fluorescence, damage, and resistance level of each single cell. The LSC enables an automated method for cell recognition and evaluation of the comets, thus providing quantitative information about nuclear DNA damage (51).

The LSC includes many of the components and analytical processes of flow cytometry, integrated with a conventional, fluorescence-adapted microscope and imaging system (52) (Fig. 18). In common with some flow cytometers, the LSC measures multicolor fluorescence, up to eight fluorochromes are currently possible (four with each laser) and light scatter on a single cell basis. Also like flow cytometry, Laser Scanning Cytometry yields high-content stoichiometric data on heterogeneous populations of large numbers of cells.

Laser Scanning Cytometry has an advantage over flow cytometry when small sub-populations of cells are being examined and morphological information as well as phenotypic information is required. The method is slide-based, therefore there is less cell loss compared with the repeated centrifugations done in flow cytometry. For our work an important and unique

feature of the LSC is cells of interest may be re-located, re-visualized, re-stained, re-measured and photographed repeatedly. The LSC offers a unique range of practical applications including: the study of the cell cycle, cell proliferation, apoptosis, immunophenotyping, enzyme kinetics, cytogenetics including FISH studies, and cell-cell interaction (53).

A prototype instrument was first described in 1991 (54), and a refined model entered commercial production in 1996, and since that time many instruments have been placed in research centers worldwide. The manufacturer of the LSC is the CompuCyte Corporation, in Cambridge, MA, United States. The computer program for the data analysis is WinCyte 3.1 software for Windows. The software is used to obtain statistical data on the individual cells. Data is collected and displayed in the conventional graphical forms used in flow cytometry. Data is displayed as either a scattergram (two selected features, one on the X-axis and the other on the Y-axis) or a histogram (frequency distribution with one of the features on the X-axis). Fig. 19 shows an example of a scattergram and histogram. The WinCyte Software has three file formats for acquiring and analyzing data (.PRO file, DPR file, and FCS file). Each of these extensions is preceded by a unique filename. The definitions for these files are described in the CompuCyte LSC Manual. A file with a .PRO (short for protocol) extension is referred to as an instrument settings file that stores the setup of the LSC for a specific application. The .DPR file is a display file that saves the screen information such as the diagrams. The .FCS file is saved in list mode with a .FCS extension and contains the instrument settings file (.Pro file) in its text section. It is compatible to the FCS 3.0 files used in Flow Cytometry. Data from both the slide and the saved .FCS file can be analyzed interchangeably and a .FCS file can be saved in Text format for further data analysis.

There are multiple techniques for preparing the cells for scanning on the LSC. Samples from single cell suspensions may be prepared by cytospin or drop preparation. Note that cells from cytospin preparations will show dramatical alterations in morphology. For antibody staining it is better to label cells in solution prior to slide preparation. For this method there is no requirement for cell fixation on the slide and fluorescence signals may be better preserved. The method we used adheres cells to a microscope slide by mixing them in agarose gel and adding the mixture to the slide.

A review by Darzynkiewicz et al. (53) gives an excellent explanation of the applications that are unique to the machine. The LSC is designed around a standard Olympus BX50 fluorescence microscope. The microscope is an integral part of the instrument and provides essential mechanical and optical components (53). The glass slide is placed on the microscope stage; the cells are excited by a laser beam that rapidly scans the microscope slide. The machine consists of beams from two lasers (488 nm Argon and 546 nm helium neon (HeNe) spatially merged by a set of dichroic mirrors (52). The path the laser beam follows is onto the computer-controlled oscillating (350 Hz) mirror, directed through the epi-illumination port of the microscope, and lastly the beams are imaged through the objective lens onto the slide (53) (Fig. 20). The laser beams rapidly sweep the area under the lens.

Light scattered by the cells is imaged by the condensor lens and recorded by scatter sensors. Fluorescence emitted by the specimen is collected by the objective lens and is partially directed to the CCD camera for imaging. Another part of fluorescence light is directed through the scan lens to the scanning mirror. Upon reflection, it passes through a series of dichroic mirrors and optical interference filters to reach one of the four photomultiplers (Green, Orange, Red, and Long Red) (53) (Fig. 20). Each photomultiplier records fluorescence at a specific

wavelength, defined by the combination of filters and dichrotic mirrors (53). In addition, the excitation light scattered by the specimen is detected by a photodiode below the object producing a signal called forward scatter (FSC), a parameter related to the morphology of the cells. There is an additional light piece that allows the observer to visualize the objects through the eyepiece of the microscope or the CCD camera.

The slides position is monitored by sensors and as the slide is being scanned the computer-controlled motorized microscope stage moves at 0.5 μm steps per laser scan, perpendicularly to the scan (55). Several thousand cells can be measured per minute with high sensitivity and accuracy. Laser Scanning Cytometry converts the light from the laser excitation into electronic analog signals. The analog signals are converted to a set of digital values called pixels (smallest unit on the display screen that can be stored, displayed, addressed) in the computer's memory. In this way a pixellated bit map on the slide is built up in 0.5μ squares. For each pixel, the fluorescence value for each measured parameter is recorded.

The user defines a threshold parameter that is used to delineate the specific fluorescence from the cells from the background fluorescence of the slide (55). The computer program (WinCyte 3.1) encircles the cells of interest by a triggering contour (red rim) and the area to be analyzed can be selected by adding an analysis (integral) contour (green rim) (Figs. 21). The level of unspecific background staining is determined for each cell by applying a background contour (two blue rims). Fig. 23 shows contours when nuclear DNA is stained with a green fluorescing dye (GST antibody). The threshold contour (T) is set on green signal to detect the nucleus, e.g. as in 22a. The integration contour (I) is then set a few pixels outside of T to insure that all of the nuclear fluorescence is measured and integrated (22a). However, when cytoplasmic fluorescence is also measured, it is set far away from T (16b). It is also possible to

separately measure nuclear and cytoplamic fluorescence as shown in 16c by setting peripheral contours (P). In each case, the background contour is automatically set outside the cell and the background fluorescence is subtracted from nuclear, cytoplasmic, or total cell fluorescence. The actual cell contours as they appear in the monitor, are shown in 22d.

Once the contouring is set up then a number of parameters are measured for each cell. The machine records the X and Y Position (relative to the LSC stage's home position), the area (number of pixels occupied by the cell), the integral value (sum of the fluorescence values for each pixel in the contour), the maximum pixel (position and value of the pixel with the highest intensity of fluorescence within the contour) and FISH integral (the integrated value of all FISH probe spots within a cell) (53). Additional factors can also be measured e.g. cell perimeter, perimeter squared, texture, peripheral integral, # of Spots (number of FISH probe spots within a cell), and time of measurement. Ratios of the respective parameters are easily preset as a new parameter, and the ratiometric data are then collected or calculated during data analysis (53). The calculated data are stored as .FCS files in the WinCyte Software.

The relocation measurement allows secondary measurements of the once probed cells, using other markers. The instrument provides the unique capability to analyze the same set of cells repeatedly, record the data in a list mode fashion including the XY position of each measured cell on the slide, and integrate ("merge") results of all sequential measurements into a single file (3, 53, 56). Therefore, it appeared to be possible to scan the slide for one fluorescent measurement, conduct lysis and electrophoresis for the comet assay, rescan the slide, and obtain cell-by-cell measurements for both endpoints.(27) The next step is to correlate the analysis of the first and second scans. The article by Li et al takes advantage of the "file merge" relocation feature of the LSC (3). They were able to correlate the supravital changes that occur in

apoptosis, namely the drop in mitochondrial transmembrane potential and generation of the reactive oxygen intermediates with features revealed by analysis of fixed cells: the cell cycle position and DNA fragmentation (3).

Other methods for comet assay analysis are more time consuming and have an inherent risk of biased selection of comets due to manual selection and characterization of comet images (51). Using the LSC enables the investigator to detect small differences in fluorescence that are not visually detectable. Basco et al. proved that the LSC was useful to examine specific markers of apoptosis to determine their relationship to DNA damage in apoptotic cells (27). For this analysis, the slides are scanned on the LSC before and after the Comet Assay to measure the fluorescence of the cells. Before conducting the Comet Assay, the cells were treated with the drug, stained with GST pi and Cytokeratin Antibodies in solution, mixed with agarose and added to the slide, and then the slides were scanned using two lasers (Green fluorescent Argon laser for GST pi and Long Red fluorescent HeNe Laser for Cytokeratin). After Comet Assay treatment, the slides were stained and then rescanned with either the Green Argon laser for the DNA stain SYBR Green I (Molecular Probes S-7567) or the Long Red HeNe laser for the monomeric cyanine nucleic acid stain To-Pro-3 (Molecular Probes T-3605). The next step is to merge the data from both of the scan files to relocate the cells. This helps to distinguish what actually is a cell apart from the background on the slide. To test if the relocation is accurate, rescan only those relocated cells and observe them visually through the CCD camera.

We applied a variation of the tail moment calculation to include the measurements from the LSC to get a value for the degree of DNA damage. For the Tail Moment Calculation, we used readings from the post electrophoresis cell values. We multiplied the calculated integral value for SYBR Green (Integral-FISH Integral/Integral) by the calculated distance value z (z =

the square root of (the difference in X Value squared + the difference in Y Value squared). We also did a dose response analysis by making a ratio histogram of Integral /FISH and setting up two different regions according to the control of the experiment. The gating was set so the undamaged region contains 95% of cells and damaged region the remaining 5% of cells. The control was used to get the percentage of damage in all other doses. If there was a successful dose response then the % of damaged cells should increase as the treatment dose increases.

Combined Immunologic and Comet Procedures. We performed the Comet Assay on patient samples after treatment with BCNU (0, 100, 300, and 1000 uM BCNU) and antibody staining with GST-pi and 488 GAR time intervals (Fig. 23). Bone marrow samples were ficolled to remove any cells that were not mononuclear cells (SOP-8 is attached in the appendices). Ficoll-Paque Plus separates mononuclear cells from other cells by providing a sterile Ficoll-sodium diatrizoate solution of the proper density, viscosity, and osmotic pressure for use in isolation procedure. After centrifugation the different cells in the sample separate into separate layers according to cell density. The most dense and smallest cells the granulocytes and erythrocytes are on the bottom layer. Ficoll-Paque Plus resides in the next layer, platelets, lymphocytes, mononuclear cells in a band as the next layer, and the top layer is plasma.

This technique worked well and visually we obtained scoreable comets on each slide under the microscope (Fig. 24). After scanning on the LSC, we calculated the Tail Moment using the calculated Z value described in the LSC section of this report, by using the intergral, FISH Intregral, and the X and Y position distance parameters for each slide. Unfortunately, levels of damage were high in the control slides (Fig. 25). In other experiments we determined that the high baselines using this procedure could be decreased by using cadmium in all steps of the GST staining procedure.

Tables 1-4 show data for two patients at three different time points in their treatment using two approaches to scoring comets, the Average Tail Moment and Percent of Damaged Cells (Fig. 26). An external control was used to set the criteria for the percent of damaged cells, as discussed above. The baselines varied widely, but control slides demonstrated a much higher level of damage compared to the previous experiment that we used to set the gating. Increasing concentrations of BCNU resulted in little increase in the tail moments, but the 300 um dose showed an increased percent of damaged cells. Cells treated with the highest dose of 1000 microM BCNU showed a decrease in damage in most of the slides. This indicated that the 1000 uM drug concentration was too high, leading to cell death and highly fragmented DNA that electrophoresed off of the slides. We therefore plan to limit analysis to the 100 and 300 uM dose.

Further analysis of this experiment indicated additional problems with the scanning procedure remained. Some slides had many cells. Overcrowding caused the LSC to detects multiple cells together or separate the comet head from the comet tail. We therefore adjusted and monitored the number of cells on the slides. A second problem was improper contouring of the cells, leading to distorted results. The high degree of sensitivity that the LSC picks up background fluorescence. Too dense cells also resulted in inaccurate measurements of the amount of DNA in the comet (and thus the percentage of DNA in the tail, or tail moment). We also developed procedures to set the threshold and minimum cell size separately for different slides to control for variability in cell size. If these parameters are set too low, background fluorescent material on the slide may be counted as a cell by the LSC. If the parameters are set too high, larger comets can be excluded from the scan. Therefore, the threshold value must be

set in the beginning of the scanning period, to fit all of the slides to be scanned for one experiment and remain the same throughout all of these slides.

To solve these problems of overcrowding and improper contouring on the LSC, we have added a smaller number of cells on the slide for the Comet Assay (10,000-20,000 cells). We have also decreased the high background fluorescence on the slide, making it easier to set the threshold level on the LSC to include only the comets in the scan.

Most recent experiments integrate all of the components needed for the clinical studies using the cell line MCF-7 Adr-R to perfect the cellular resistance part of the protocol with the new methods and slides. This multi-step procedure is presented as SOP-9. We plan to continue the project by analysis of patient samples at the various drug concentrations.

Key Research Accomplishments:

- Assays for measuring transient changes in resistance to CPB therapy following induction therapy were established using procedures that use very small numbers of cells. These have been further enhanced to allow sharing of the same cells between assays for fluorescent cellular endpoint and DNA damage. This technique will allow studies of multiple doses and replicate assays for the *in vitro* studies required for this project.

- Assays demonstrated that these procedures could be used for both lymphocytes and bone marrow cells from patients

- A modification of the SCGE assay and assays for DNA damage by biochemical and molecular methods that can be used to study transient changes in cells stored for prolonged periods

- Further assay modifications allow the same specimen to be used for cellular fluorescence and DNA damage assays, increasing capabilities and the accuracy for analyzing small number of cells.

- Additional assays for DNA modifications will allow the analysis of specimens in existing tissue repositories where sample preservation was not suitable for the SCGE approach

- Techniques for measuring DNA adducts by their interference with the polymerase chain reaction have been implemented and approaches for high-throughtput analysis by real time fluorescent monitoring were developed.

- Each of the agents in CPB produces DNA damage that can be measured with the SCG assays in a dose dependent manner

- At low doses all three agents cause a similar pattern of breakage, while at high doses their pattern of damage could be distinguished (Cisplatin contracted the cells, consistent with its ability to cause DNA crosslinks; BCNU caused a pattern

- Both lymphocytes and bone marrow cells from patients can be routinely analyzed while sharing the specimens with other assays, allowing study of changes in sensitivity in small quantities of multiple tissues

- Analytic approaches including polymerase chain reaction and chemically based approaches that use repositories of non-ideally preserved specimens are feasible

- Using a laser scanning cytometer, individual cells can be identified on slides. The characteristics of the individual cells (e.g., the presence of markers for normal or cancer cells) and cytoplasmic features (e.g. markers for apoptosis or enzymatic or other factors associated with resistance to chemotherapy) can be characterized by immunohistochemistry.

- A Assays for DNA damage can be conducted in the same cells and associations between DNA damage and cytoplasmic features determined at the individual cell level.

Reportable Outcomes (Copies are provided as the last Appendix)

Land, S.J., Bacsó, Zs., Klein, J., Eliason, J.F., and Everson R.B. Quantitative methods for examining the drug resistance phenotype of micrometastatic cancer cells in bone marrow: DNA damage in response to in vitro drug treatment. *Proceedings of the American Association for Cancer Research* 40:403,1999

Toset, A.W., Everson, R.B.: Measurement of DNA repair in BRCA1 and BRCA2 deficient human cell lines with the Single Cell Gel Electrophoresis (SCGE) assay. *Proceedings of the American Association for Cancer Research* 41: 335, 2000

Everson, R.B., Land, S.J., Eliason, J.F., Klein, J.L., and Baynes R.D. Transient resistance to high-dose chemotherapy: Evaluation of the use of DNA-damage assays to optimize treatment schedule. *Proceedings of the Department of Defense Breast Cancer Research Program Meeting: Era of Hope* Vol. II: 690, 2000

Bacso, Z., Everson, R. B., and Eliason, J. F. The DNA of annexin V-binding apoptotic cells is highly fragmented. *Cancer Research* 60: 4623-4628., 2000.

Fellowship/Degrees Obtained:

Master of Science in Basic Medical Science and Summer Research Experience Fellowship Program were award to Rosemarie Chirco based on work for this project.

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Conclusions

For drugs that interact with DNA, measures of DNA damage can assess the intracellular availability of active drug at a critical molecular target. Measurements of DNA damage should reflect the integrated effect of all resistance factors, including both recognized mechanisms and uncharacterized mechanisms. Thus, molecular measures of DNA damage could provide an important tool for elucidating the time course of complex changes in resistance factors. Motivated by a recent clinical trial that demonstrated better survival when the interval between induction chemotherapy and high dose therapy was prolonged, this project is using measures of DNA damage to determine whether induction chemotherapy causes transient changes in resistance. Findings indicate cyclophosphamide, cis-platin, and BCNU each produce DNA damage that can be measured in a dose dependent manner. Using a laser scanning cytometer, individual cells can be identified on slides. The characteristics of the individual cells (e.g., the presence of markers for normal or cancer cells) and cytoplasmic features (e.g. markers for apoptosis or enzymatic or other factors associated with resistance to chemotherapy) can be characterized by immunohistochemistry. Assays for DNA damage can be conducted in the same cells and associations between DNA damage and cytoplasmic features determined at the individual cell level. Using a laser scanning cytometer, individual cells can be identified on slides. The characteristics of the individual cells (e.g., the presence of markers for normal or cancer cells) and cytoplasmic features (e.g. markers for apoptosis or enzymatic or other factors associated with resistance to chemotherapy) can be characterized by immunohistochemistry. Assays for DNA damage can be conducted in the same cells and associations between DNA damage and cytoplasmic features determined at the individual cell level.

Although designed around an observational study of high dose chemotherapy, all data obtained from this project are equally applicable to understanding resistance in conventional dose chemotherapy. Our work toward development of measurements of DNA damage and molecular doses for clinical oncology will provide a rapid way to measure the effects of alternative treatment programs (2, 3). These measurements would provide an approach to developing optimal treatment protocols using fewer patients in a much more rapid time frame than could be achieved using only clinical outcomes. We are confident it will provide useful contributions to defining standards of care in the future.

References

1. Peters, W. P., Jones, R.B., and Vredenburgh, J. A large, prospective, randomized trial of high-dose combination alkylating agent (CPB) with autologous cellular support (ABMS) as consolidation for patients with metastatic breast cancer achieving complete remission after intensive doxorubicin-based induction therapy (AFM). Proc Am Soc Clin Oncol., *15:* 149a, 1996.

2. Everson, R. B., Land, S.J., Eliason, J.F., Klein, J.L., and Baynes, R.D. Transient resistance to high-dose chemotherapy: Evaluation of the use of DNA-damage assays to optimize treatment schedule. Accepted for presentation at Department of Defense Breast Cancer Research program "Era of Hope Meeting June 8-12, 2000.

3. Land, S. J., Basco, Zs., Klein, J., Eliason, J.F., and Everson, R.B. Quantitative methods for examing the drug resistance phenotype of micrometastatic cancer cells in bone marrow: DNA damage in response to in vitro drug treatment. Proceedings of the American Association for Cancer Research, *40:* 403, 1999.

4. Baynes, R. D., Dansey, R. D., Klein, J. L., Hamm, C., Campbell, M., Abella, E., and Peters, W. P. High-dose chemotherapy and hematopoietic stem cell transplantation for breast cancer: past or future? Semin Oncol, *28:* 377-388., 2001.

5. Peters, W. P., Dansey, R. D., Klein, J. L., and Baynes, R. D. High-dose chemotherapy and peripheral blood progenitor cell transplantation in the treatment of breast cancer. Oncologist, *5:* 1-13, 2000.

6. O'Shaughnessy, J. High Dose Chemotherapy for Breast Cancer: Taking Stock. Oncologist, *5:* 14-17, 2000.

7. Antman, K. H. Overview of the six available randomized trials of high-dose chemotherapy with blood or marrow transplant in breast cancer. J Natl Cancer Inst Monogr, *30:* 114-116, 2001.

8. Rodenhuis, S. The Status of High-Dose Chemotherapy in Breast Cancer. Oncologist, *5:* 369-375, 2000.

9. Ye, Q. and Bodell, W. J. Detection of N7-(2-hydroxyethyl)guanine adducts in DNA and 9L cells treated with 1-(2-chloroethyl)-1-nitrosourea. J Chromatogr B Biomed Sci Appl, *694:* 65-70., 1997.

10. Grimaldi, K. A., McAdam, S. R., and Hartley, J. A. PCR-based assays for strand-specific measurement of DNA damage and repair. II. Single-strand ligation-PCR. Methods Mol Biol, *113:* 241-255, 1999.

11. Grimaldi, K. A., Bingham, J. P., and Hartley, J. A. PCR-based assays for strand-specific measurement of DNA damage and repair. I. Strand-specific quantitative PCR. Methods Mol Biol, *113:* 227-240, 1999.

12. Grimaldi, K. A., McAdam, S. R., Souhami, R. L., and Hartley, J. A. DNA damage by anti-cancer agents resolved at the nucleotide level of a single copy gene: evidence for a novel binding site for cisplatin in cells. Nucleic Acids Res, *22:* 2311-2317., 1994.

13. Grimaldi, K. A., Bingham, J. P., Souhami, R. L., and Hartley, J. A. DNA damage by anticancer agents and its repair: mapping in cells at the subgene level with quantitative polymerase chain reaction. Anal Biochem, *222:* 236-242., 1994.

14. Pfeifer, G. P., Denissenko, M. F., and Tang, M. S. PCR-based approaches to adduct analysis. Toxicol Lett, *102-103:* 447-451., 1998.

15. Collins, A. R., Dobson, V. L., Dusinska, M., Kennedy, G., and Stetina, R. The comet assay: what can it really tell us? Mutat Res, *375:* 183-193., 1997.

16. Singh, N. P. e. a. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res, *1988:* 184-191, 1988.

17. Ryberg, B. and Johanson, K. J. Estimation of DNA strand breaks in single mammalian cells, p. 465-468. New York: Academic Press, 1978.

18. Östling, O. a. J., K.J. Microelectrophoresis study of radiation-induced DNA damage in individual mammalian cells. Biochem and Biophy Res Comm, *123:* 291-298, 1984.

19. Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C., and Sasaki, Y. F. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen, *35:* 206-221, 2000.

20. Rojas, E., Lopez, M. C., and Valverde, M. Single cell gel electrophoresis assay: methodology and applications. J Chromatogr B Biomed Sci Appl, *722:* 225-254., 1999.

21. Olive, P. L. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. Int J Radiat Biol, *75:* 395-405., 1999.

22. McKelvey-Martin, V. J., Green, M. H., Schmezer, P., Pool-Zobel, B. L., De Meo, M. P., and Collins, A. The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res, *288:* 47-63., 1993.

23. Fairbairn, D. W., Olive, P. L., and KL, O. N. The comet assay: a comprehensive review. Mutat Res, *339:* 37-59, 1995.

24. Green, M. H., Lowe, J. E., and Delaney, C. A., and Green, I. C. Comet assay to detect nitric oxide-dependent DNA damage in mammalian cells. Methods Enzymol, *269:* 243-266, 1996.

25. Olive, P. L., Banath, J. P., and Durand, R. E. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat Res, *122:* 86-94., 1990.

26. Olive, P. L., Banath, J. P., and Durand, R. E. Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells. J Natl Cancer Inst, *82:* 779-783., 1990.

27. Bacso, Z., Everson, R. B., and Eliason, J. F. The DNA of annexin V-binding apoptotic cells is highly fragmented. Cancer Res, *60:* 4623-4628, 2000.

28. Kopjar, N., Garaj-Vrhovac, V., and Milas, I. Assessment of chemotherapy-induced DNA damage in peripheral blood leukocytes of cancer patients using the alkaline comet assay. Teratog Carcinog Mutagen, *22:* 13-30, 2002.

29. Zwelling, L. A. Consequences of the resistance to drug-induced topoisomerase II-mediated DNA cleavage. Toxicol Lett, *67:* 231-236., 1993.

30. Trimmer, E. E. and Essigmann, J. M. Cisplatin. Essays Biochem, *34:* 191-211, 1999.

31. Pieper, R. O., Noftz, S. L., and Erickson, L. C. In vitro transcription termination by N,N'-bis(2-chloroethyl)-N- nitrosourea-induced DNA lesions. Mol Pharmacol, *47:* 290-295., 1995.

32. Doehmer, J. D., S; Friedberg, T; Monier, S; Adesnik, M; Glatt, H; Oesch, F Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B1. Proc. Natl. Acad. Sci. USA., *85,:* 5769–5773., 1988.

33. Tew, K. D., O'Brien, M., Laing, N.M., and Shen, H. Coordinate changes in expression of protective genes in drug-resistant cells. Chem Biol Interact, *11-112:* 199-211, 1998.

34. Woods, J. S., Kavanagh, T.J., Corral, J., Reese, A.W., Diaz, D., and Ellis, M.E. The role of glutathione in chronic adaptation to oxidative stress: studies in a normal rat kidney epithelial (NRK52E) cell model of sustained upregulation of Glutathione biosynthesis. Toxicology and Applied Pharmacology, *160:* 207-216, 1999.

35. O'Brien, M., Kruh, G.D., and Tew, K.D. The influence of coordinate overexpression of glutathione phase II detoxification gene products on drug resistance. J Pharm and Exp Therap, *294:* 480-487, 2000.

36. Alpert, L. C., Schecter, R.L., Berry, D.A., Melnychuk, D., Peters, W.P., Caruso, J.A., Townsend, A.J., and Batist, G. Relation of glutathione S-transferase alpha and mu isoforms to response to therapy in human breast cancer. Clinical Cancer Research, *3:* 661-667, 1997.

37. Smith, M. T., Evans, C. G., Doane-Setzer, P., Castro, V. M., Tahir, M. K., and Mannervik, B. Denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea by class mu glutathione transferases and its role in cellular resistance in rat brain tumor cells. Cancer Res, *49:* 2621-2625., 1989.

38. Dirven, H. A., van Ommen, B., and van Bladeren, P.J. Involvement of human glutathione S-transferase isoenzymes in the conjugation of cyclophosphamide metabolites with glutathione. Cancer Research, *54:* 6215-6220, 1994.

39. Townsend, A. J., Tu, C.P.D., and Cowan, K.H. Expression of human mu or alpha class glutathione S-transferases in stably transfected human MCF-7 breast cancer cells- effect on cellular sensitivity to cytotoxic agents. Molecular Pharmacology, *41:* 230-236, 1992.

40. Lai, G. M., Moscow, J. A., Alvarez, M. G., Fojo, A. T., and Bates, S. E. Contribution of glutathione and glutathione-dependent enzymes in the reversal of adriamycin resistance in colon carcinoma cell lines. Int J Cancer, *49:* 688-695., 1991.

41. Kramer, R. A., Zakher, J., and Kim, G. Role of the glutathione redox cycle in acquired and de novo multidrug resistance. Science, *241:* 694-697, 1998.

42. Hosking, L. K., Whelan, R. D., Shellard, S. A., Bedford, P., and Hill, B. T. An evaluation of the role of glutathione and its associated enzymes in the expression of differential sensitivities to antitumour agents shown by a range of human tumour cell lines. Biochem Pharmacol, *40:* 1833-1842., 1990.

43. Silvestrini, R., Veneroni, S., Benini, E., Daidone, M.G., Luisi, a., Leutner, M., Kenda, R., Zucali, R., and Veronesi, U. Expression of p53, glutathione S-transferase-pi, and bcl-2 proteins and benefit from adjuvant radiotherapy in breast cancer. J Natl Cancer Inst, *89:* 639-645, 1997.

44. Braun, S., Pantel, K, Muller, P, Janni, W., Hepp, F., Kentenich, C.R., Gastroph, S., Wischnik, A., Dimpfl, T., Kindermann, G., Riethmuller, G., and Schlimok, G. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N Engl J Med, *342:* 525-533, 2000.

45. Borgen, E., Naume, B., Nesland, J.M., Kvalheim, G., Fodstad, O., Diel, I., Solomayer, E.F., Theocharous, P., Coomber, R.C., Smith, B.M., Wunder, E., Marolleau, J.P., Garcia, J., and Pantel, K. Standardization of the immunocytochemical detection of cancer cells in bone marrow and blood. I. Establishment of objective criteria for the evaluation of immunostained cells. Cytotherapy, *1:* 377-388, 1999.

46. Broers, J. L. V., Ramaekers, F.C.S., Rot, M.K., Oostendorp, T., Huysmans, A., van Muijen, G.N.P., Wagenaar, S.Sc., and Vooijs, G.P. Cytokeratins in different types of human lung cancer as monitored by chain-specific monoclonal antibodies. Cancer Research, *48*, 1988.

47. Werther, K., Normark, M., Hansen, B.F., Brunner, N, and Nielsen, H.J. The use of CELLection Kit TM in the isolation of carcinoma cells from mononuclear cell suspensions. Journal of Immunological Methods, *238:* 133-141, 2000.

48. Pantel, K., Cote, R.J., and Fodstad, O Detection and Clinical Importance of Micrometastatic Disease. Journal of the National Cancer Institute, *91:* 1113-1124, 1999.

49. Hellman, B., Vaghef, H., and Bostrom, B. The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay. Mutat Res, *336:* 123-131, 1995.

50. Olive, P. L., Banath, J. P., and Durand, R. E. Detection of subpopulations resistant to DNA-damaging agents in spheroids and murine tumours. Mutat Res, *375:* 157-165, 1997.

51. Petersen, A. B., Gniadecki, R., and Wulf, H. C. Laser scanning cytometry for comet assay analysis. Cytometry, *39:* 10-15., 2000.

52. Rew, D., Woltman, G., and Wardlaw, A. Laser-Scanning Cytometry. The Lancet, *353:* 255-256, 1999.

53. Darzynkiewicz, Z., Bedner, E., Li, X., Gorczyca, W., and Melamed, M. R. Laser-scanning cytometry: A new instrumentation with many applications. Exp Cell Res, *249:* 1-12., 1999.

54. Kamentsky, L. A. and Kamentsky, L. D. Microscope based multiparameter laser scanning cytometry yielding data comparable to flow cytometry data. Cytometry, *12:* 381-387, 1991.

55. Kamentsky, L. A., Kamentsky, L. D., Fletcher, J. A., Kurose, A., and Sasaki, K. Methods for automatic multiparameter analysis of fluorescence in situ hybridized specimens with a laser scanning cytometer. Cytometry, *27:* 117-125., 1997.

56. Clatch, R. J., Foreman, J.R., and Walloch, J.L. Simplifies immunophenotypic analysis by laser scanning cytometry. Cytometry, *34:* 3-16, 1998.

Appendicies

Appendices

A. List of Figures

21. Contouring Cells During The First Scan After GST Staining
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24. Variability in Comet Size for Each Drug Dose For Patient 1 – Before Drug Induction Time Sample
25. Patient Samples Without Micrometastasis to the Bone Marrow: Data for Patient 2
26. Tables 1-4 Data for the Average Tail Moment and Percent of Damaged Cells for Patient Samples
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Fig. 1 AFM Randomized Study: Trial Design and Results

The AFM Randomized Clinical Trial showed patients who received high-dose cyclophosphamide, cisplatin, BCNU (CPB) therapy at relapse after a complete response (CR) to induction therapy had a two-fold greater overall survival than patients who received high dose therapy immediately after induction therapy. Fig. A below shows the design of the trial. PR stand for partial response.

In the AFM Randomized Trial induction therapy was with adriamycin, 5-FU and methotrexate (AFM) and high dose therapy consisted of cyclophosphamide, cisplatin, and BCNU (CPB). Immediate treatment was within one week, while treatment after relapse started a median of 21 weeks after relapse (Fig. B below).

Overall survival curves for AFM randomized trial. Solid line, CR patients receiving CPB immediately. Long dashed line, CR patients receiving CPB at relapse; short dashed line, PR patients receiving CPB immediately

Fig. a

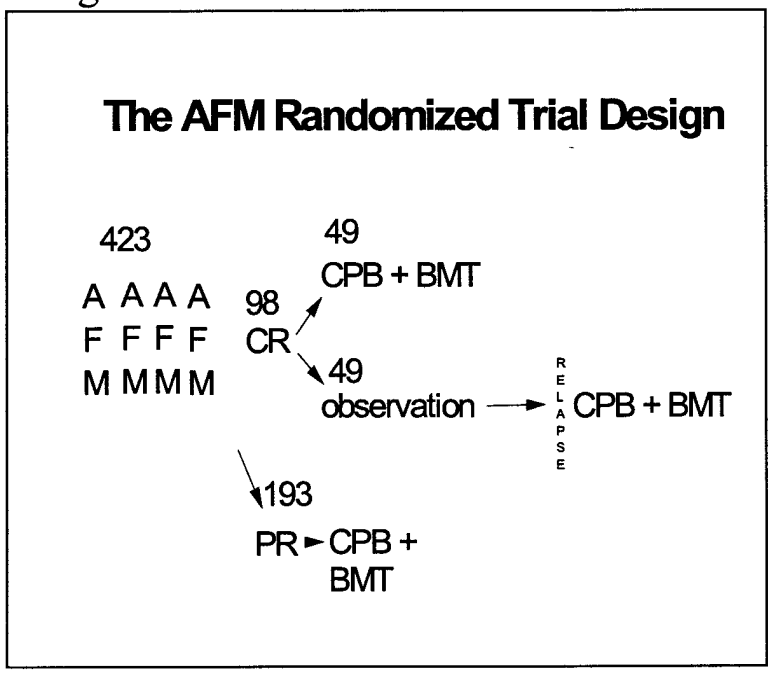


Fig.b

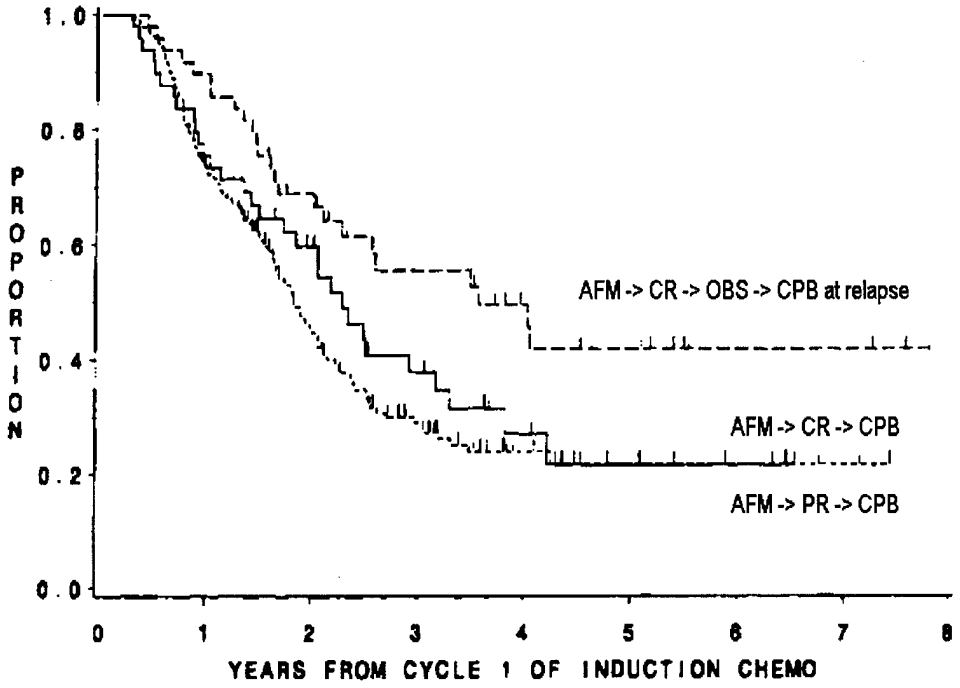
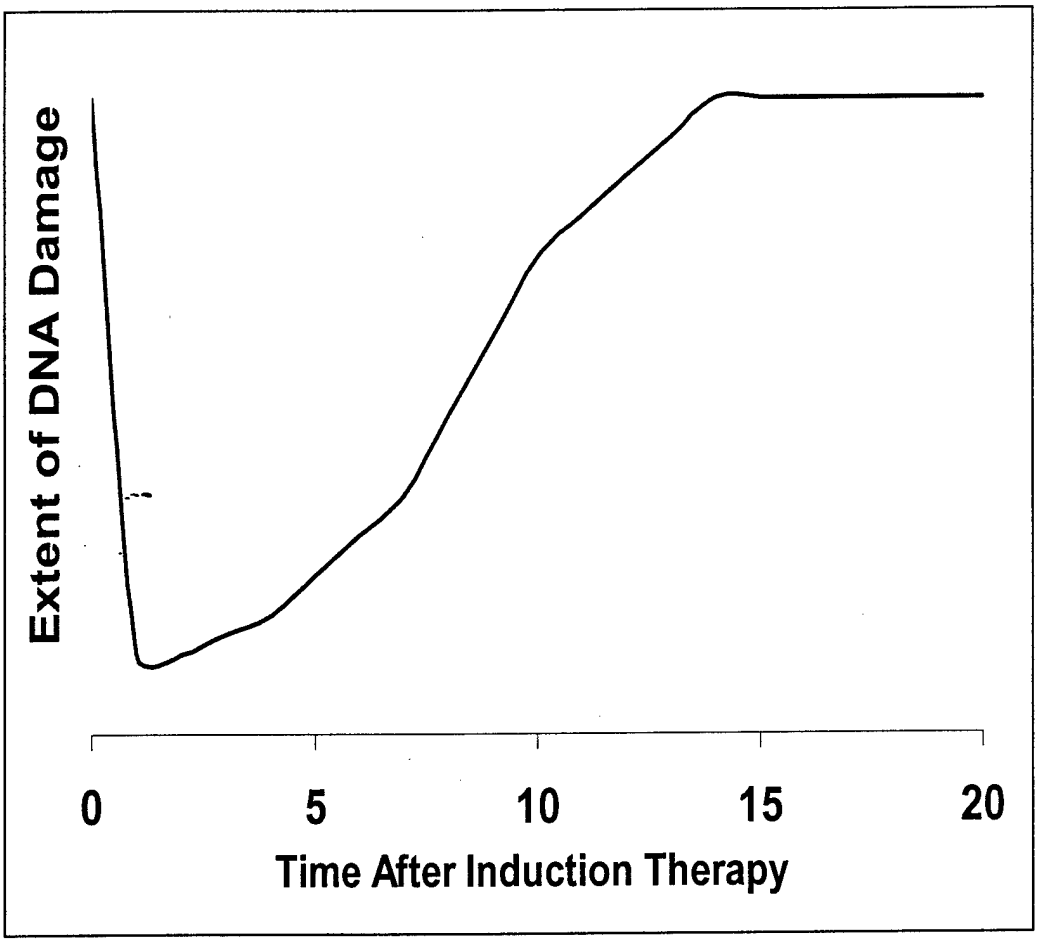


Fig. 2. Hypothetical Changes in DNA Damage after Induction Therapy

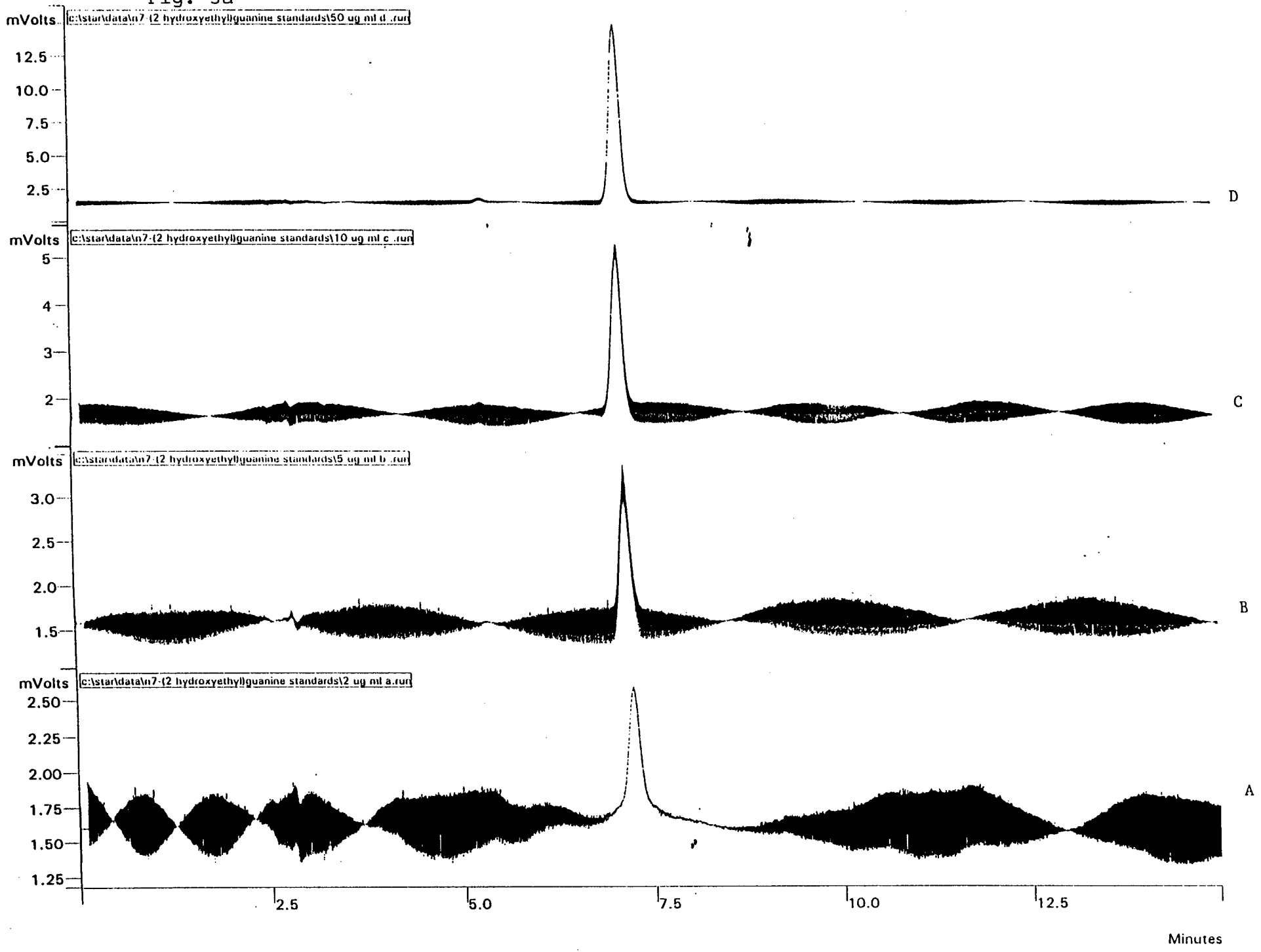


My hypothesis is that a transient change in cellular resistance to high dose therapy is brought on by induction therapy. As Illustrated above, before induction chemotherapy resistance to chemotherapy may be low so that damage induced by a given exposure to high-dose therapy would be high. Induction therapy might then increase resistance, resulting in less DNA damage from the same high-dose therapy. If the induced resistance is transient, levels of DNA damage will increase as time passes after therapy. Thus measurements of levels of damage after therapy could suggest the optimal interval between induction therapy and high-dose therapy – **a point where induced damage had returned to a high level but the disease had not been allowed to progress.** We developed approaches to that question by analysis of DNA damage using the Comet Assay.

Fig. 3a and 3b

- The following two figures illustrate HPLC analysis of DNA adducts for N7-(2 hydroxyethyl)guanine (N7-HOEtG) standards. Sodium acetate buffer at pH 5.4 was used as mobile phase A and methanol was used as mobile phase B. The N7-HOEtG standards were run with the following HPLC isocratic program: 90% A and 10% B for 12 min. N7-HOEtG in 15 ul mobile phase was injected at concentrations of 2 ug/ml (A), 5 ug/ml (B), 10 ug/ml (C), 50 ug/ml (D).

Fig. 3a


mVolts
c:\star\data\n7-(2 hydroxyethyl)guanine standards\50 ug ml d .run
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10.0
7.5
5.0
2.5
D
mVolts
c:\star\data\n7-(2 hydroxyethyl)guanine standards\10 ug ml c .run
5
4
3
2
C
mVolts
c:\star\data\n7-(2 hydroxyethyl)guanine standards\5 ug ml b .run
3.0
2.5
2.0
1.5
B
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2.50
2.25
2.00
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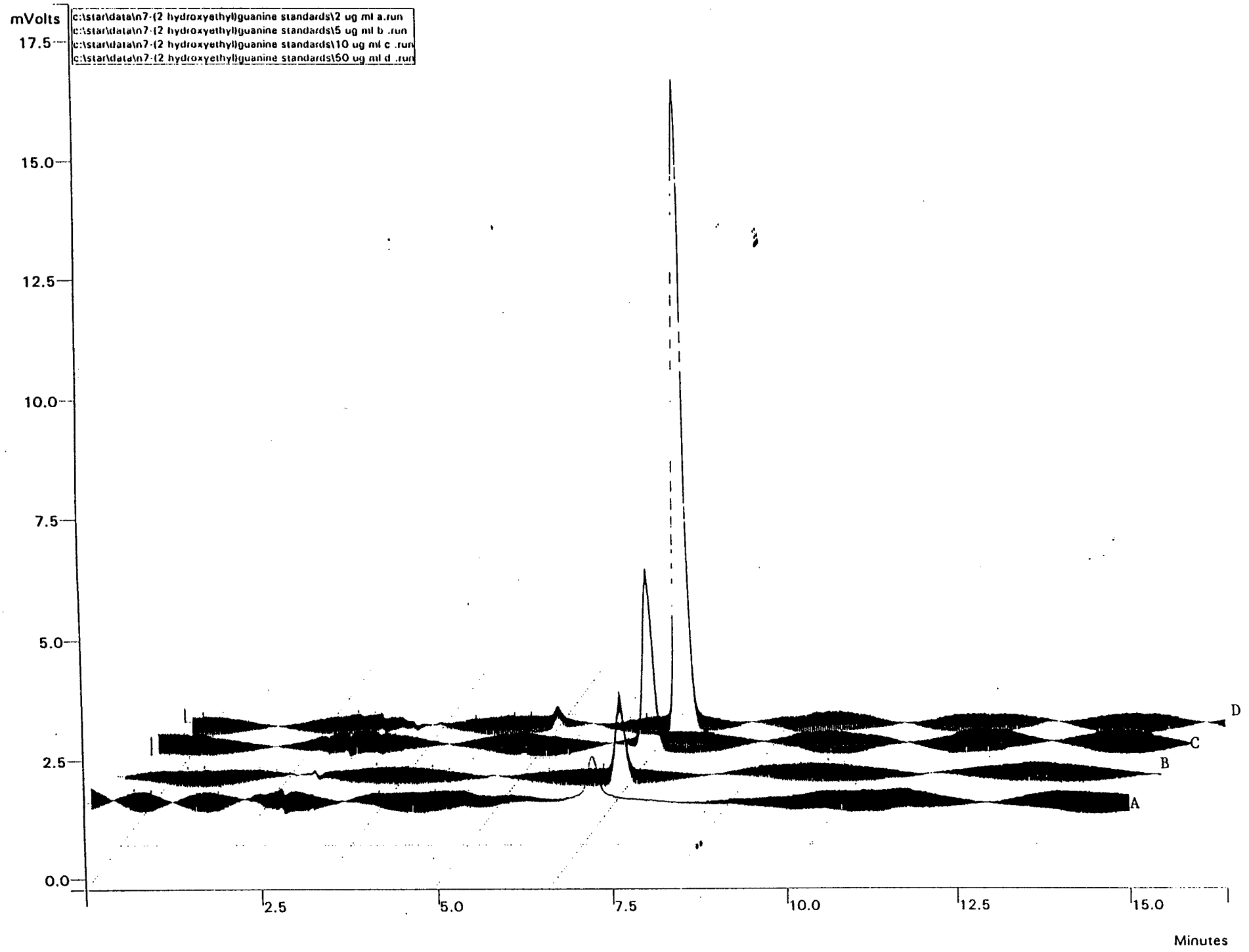
1.25
A
2.5
5.0
7.5
10.0
12.5
Minutes

Fig. 3b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c:\star\data\n7-(2 hydroxyethyl)guanine standards\50 ug ml d .run
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
A
B
C
D
2.5
5.0
7.5
10.0
12.5
15.0
Minutes

Fig. 4 Single Cell Gel Electrophoresis or Comet Assay

The SCGE assay is conducted as follows:

-- Isolate cells
-- Embed cells in agarose
-- Lyse cell to remove membrane and proteins leaving DNA
-- Denature DNA with high pH
-- Electrophorese cells
-- Neutralize, dry, and stain (SYBR Green)
-- Quantify damage by image analysis

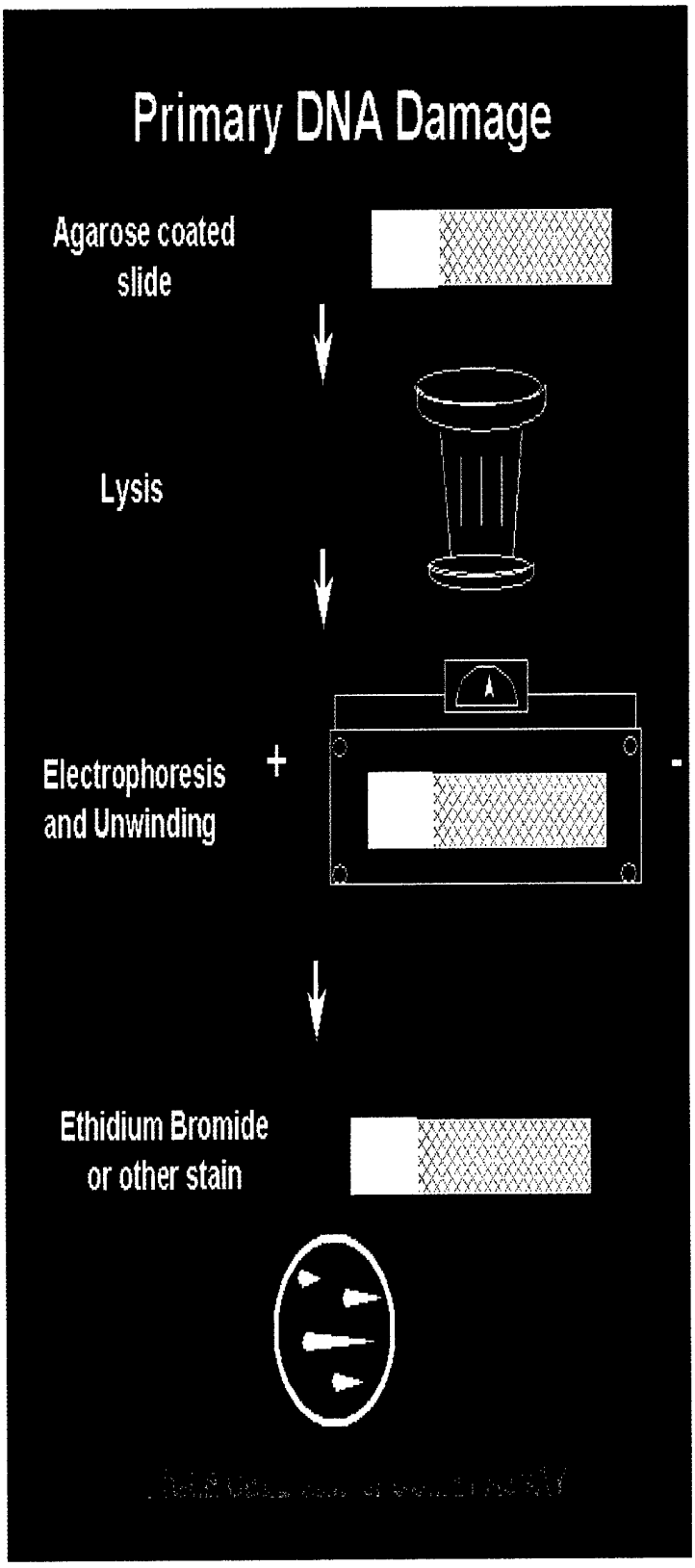
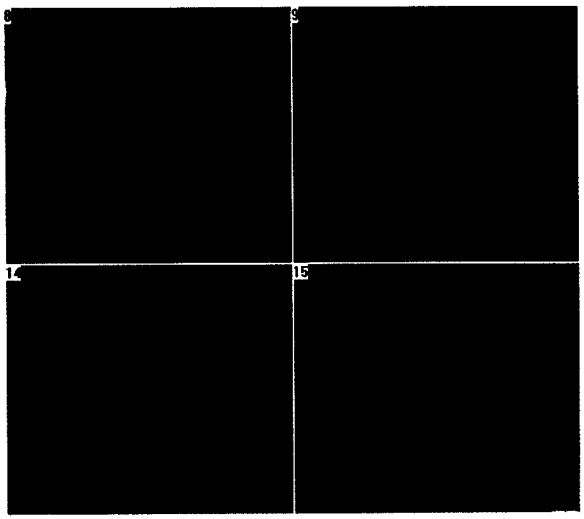


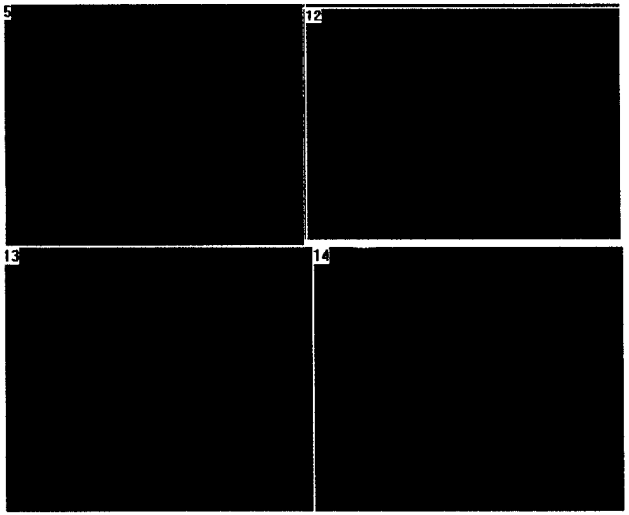
Fig. 5 DNA Migration Patterns of Treated Cell Lines

Fig. 4a. MCF-7 Cells treated with 0 μM BCNU. This is the control slide, you can see that there is no damage.

Fig. 4b. MCF-7 Cells treated with 100 μM BCNU. Notice that there is less space between the head and the tail of the comet.

Fig. 4c MCF-7 Cells treated with 300 μM BCNU. Notice the large apoptotic comets (large space between the head and tail of the comets).





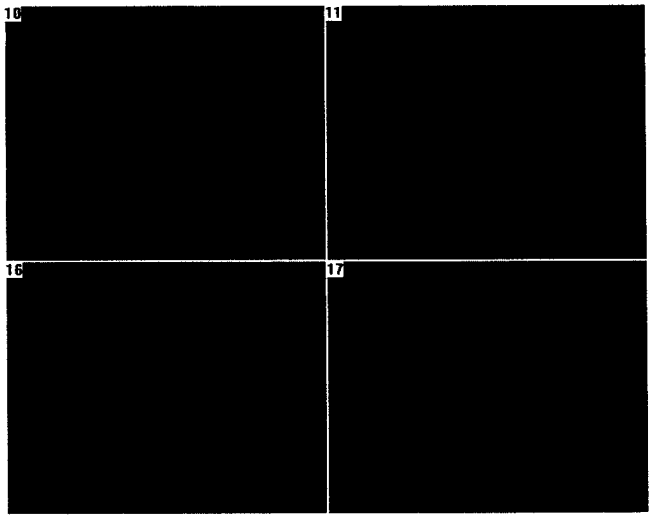


Fig 6. Patterns of Damage and Dose Response Curves for Comet Assays of Agents in CBP.

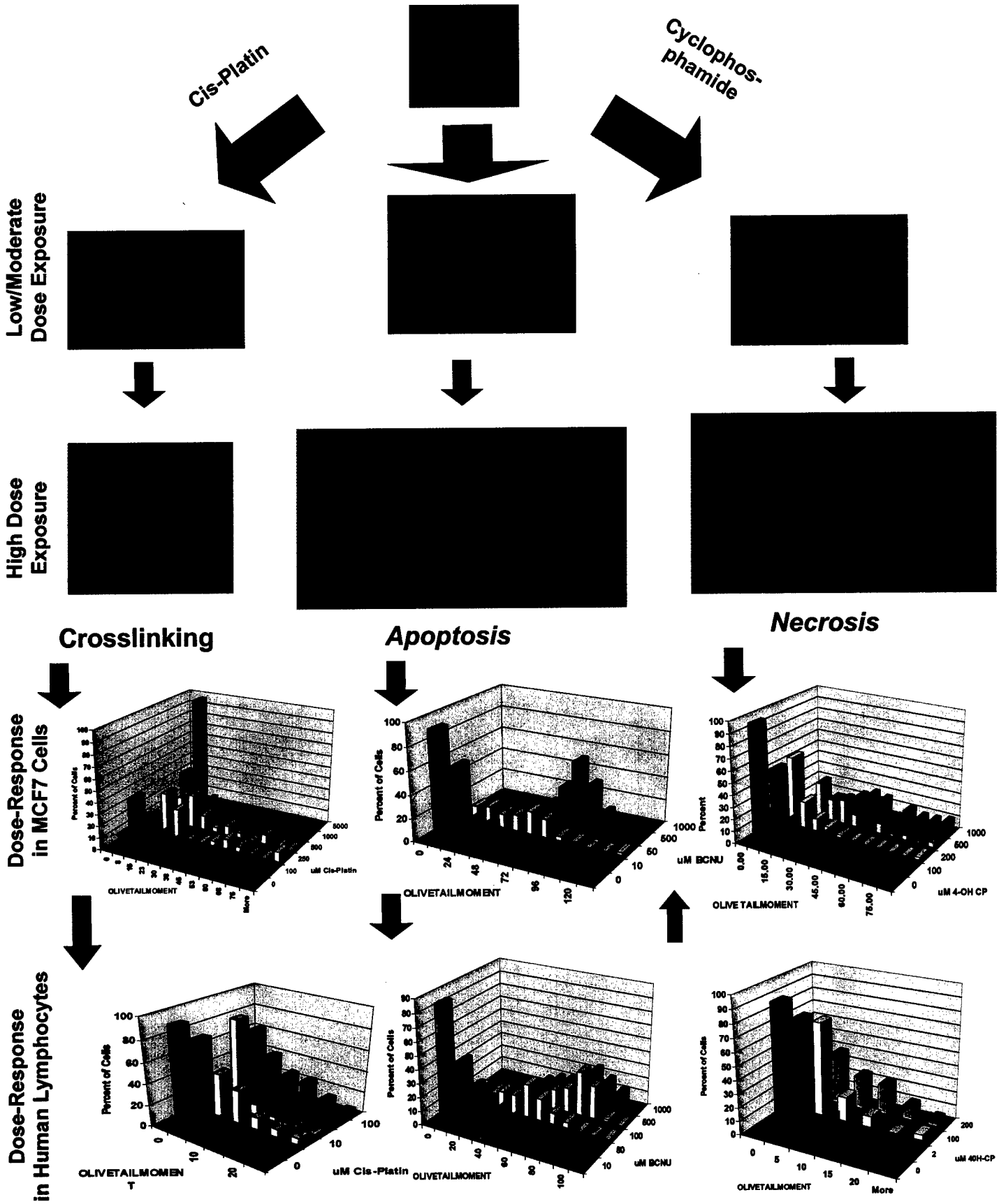


Fig. 7 MCF-7 Cells Treated with BCNU Before and After Electrophoresis

Fig. 7a. Before Electrophoresis

Fig. 7b. After Electrophoresis

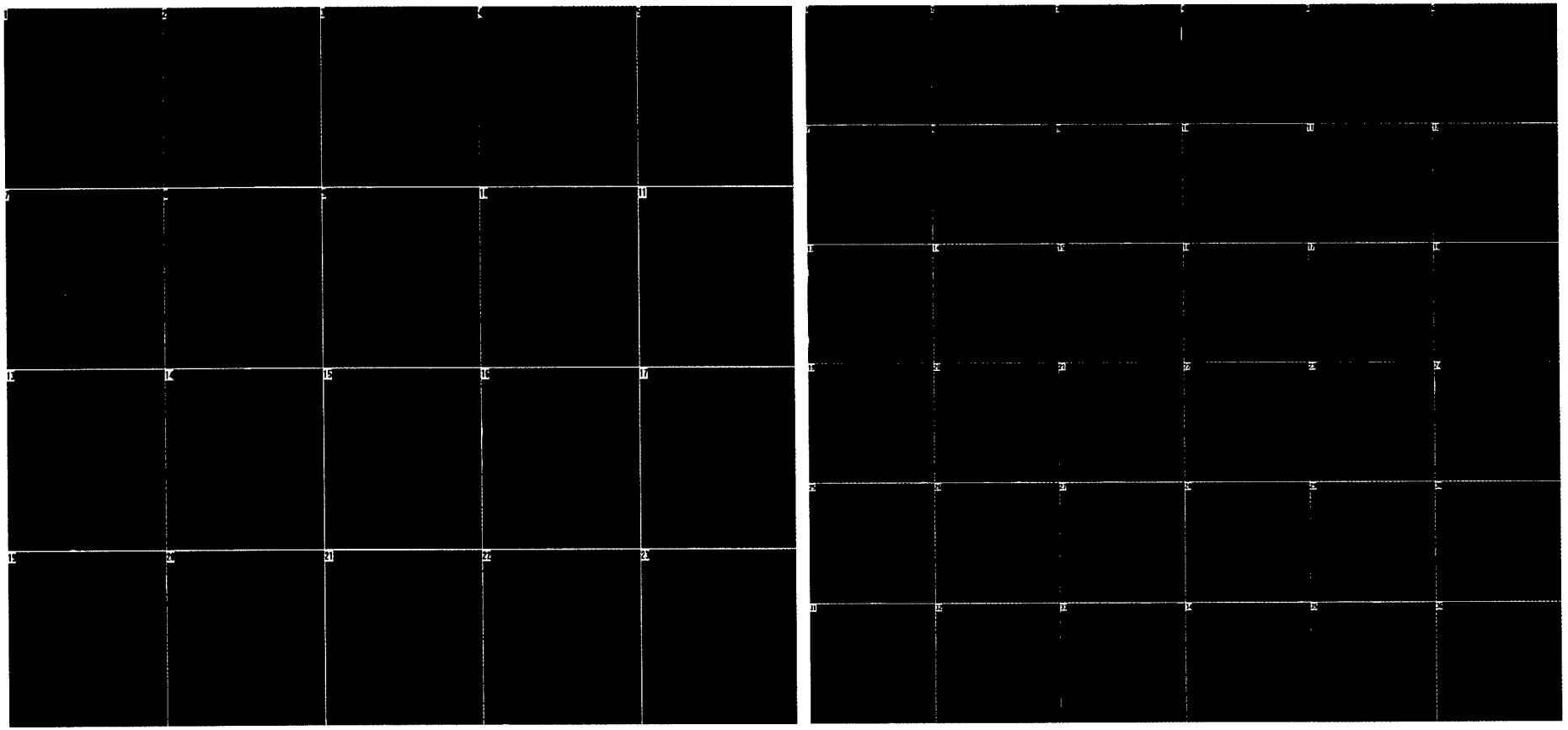
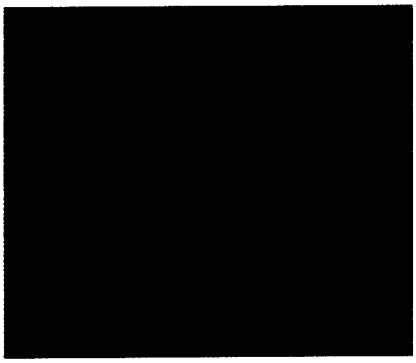


Fig. 7 MCF-7 Adr-R Cells treated with 300 μM BCNU and stained with GST-π and 488 Goat Anti-Rabbit Antibodies before electrophoresis (Fig. a). After electrophoresis the cells were stained with SYBR Green (Fig. b).

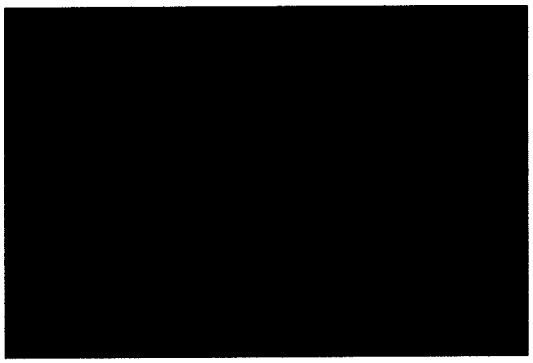
Fig. 8 Comparing the Three Different GSTs: Pi, Mu, and Alpha

GST-Pi

Before Electrophoreis 300 μM BCNU GST pi

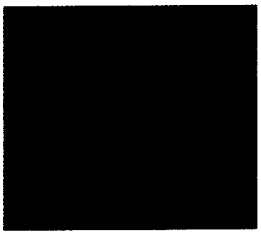


After Electrophoreis 0 μM BCNU GST pi

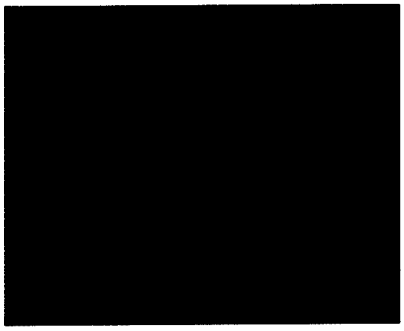


After Electrophoreis 300 μM BCNU GST pi

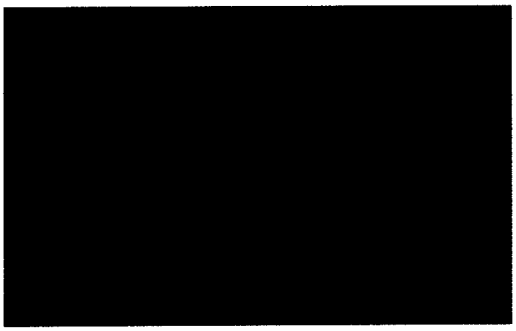
GST-Mu



Before Electrophoreis 300 μM BCNU GST mu

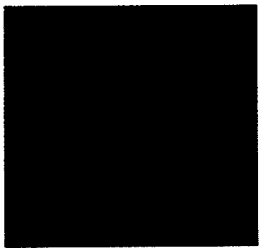


After Electrophoreis 0 μM BCNU GST mu

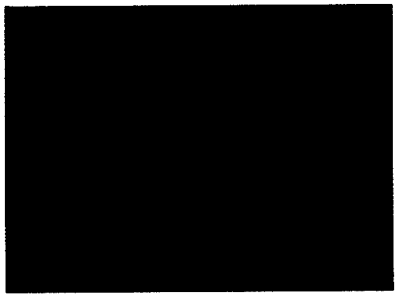


After Electrophoreis 300 μM BCNU GST mu

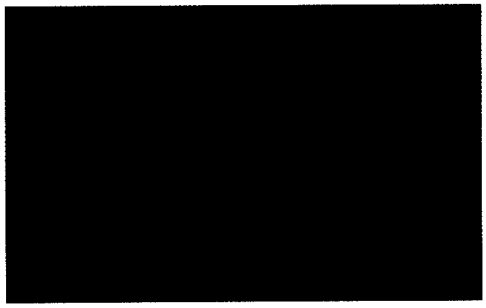
GST-Alpha



Before Electrophoreis 300 μM BCNU GST alpha



After Electrophoreis 0 μM BCNU GST mu



After Electrophoreis 300 μM BCNU GST alpha

Fig. 9 Comparison of Two Comet Assay Methods: The Original Method and the Trevigen CometSlides™ Method After Treatment with BCNU and No GST Staining

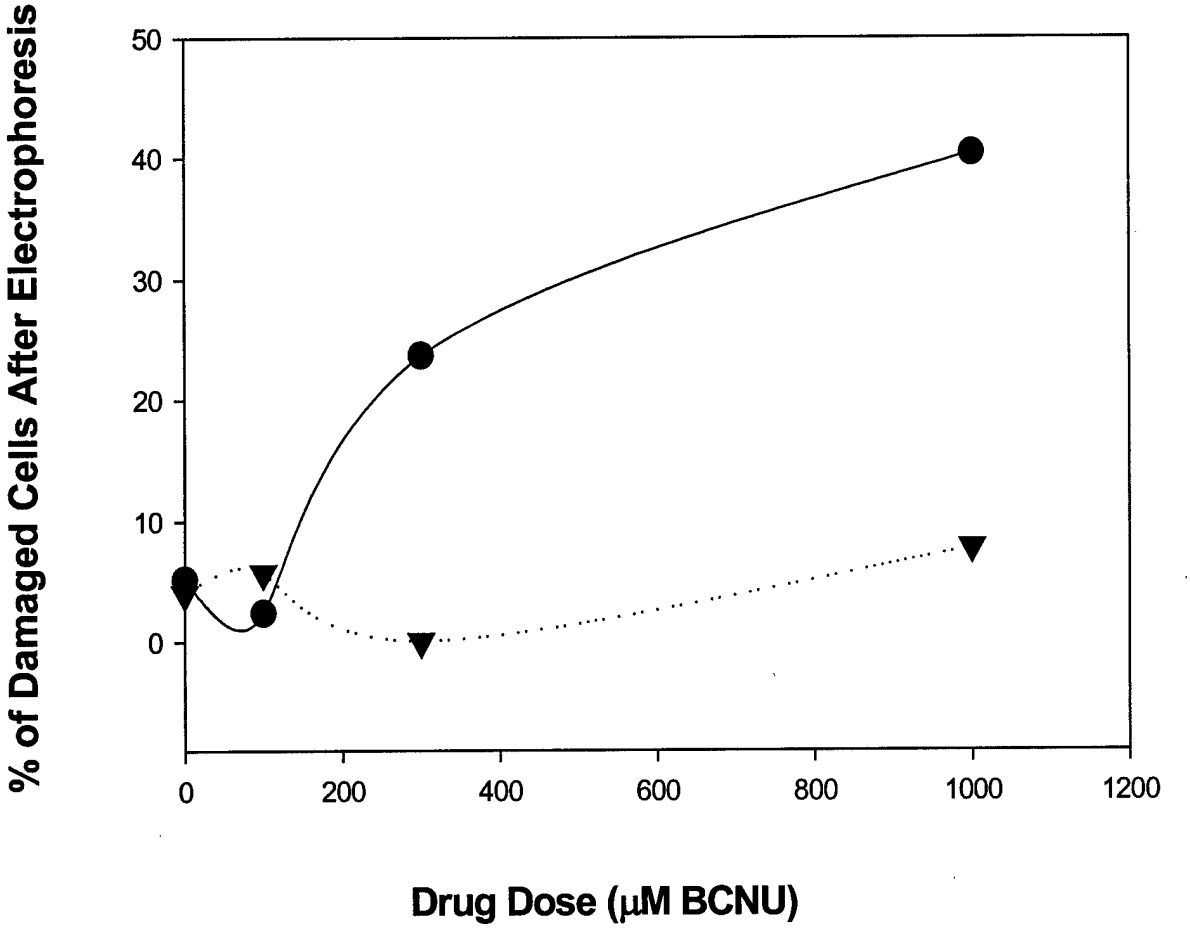


Fig. 10 Treating MCF-7 Adr-R cells with BCNU Using the Trevigen CometSlides™ Method

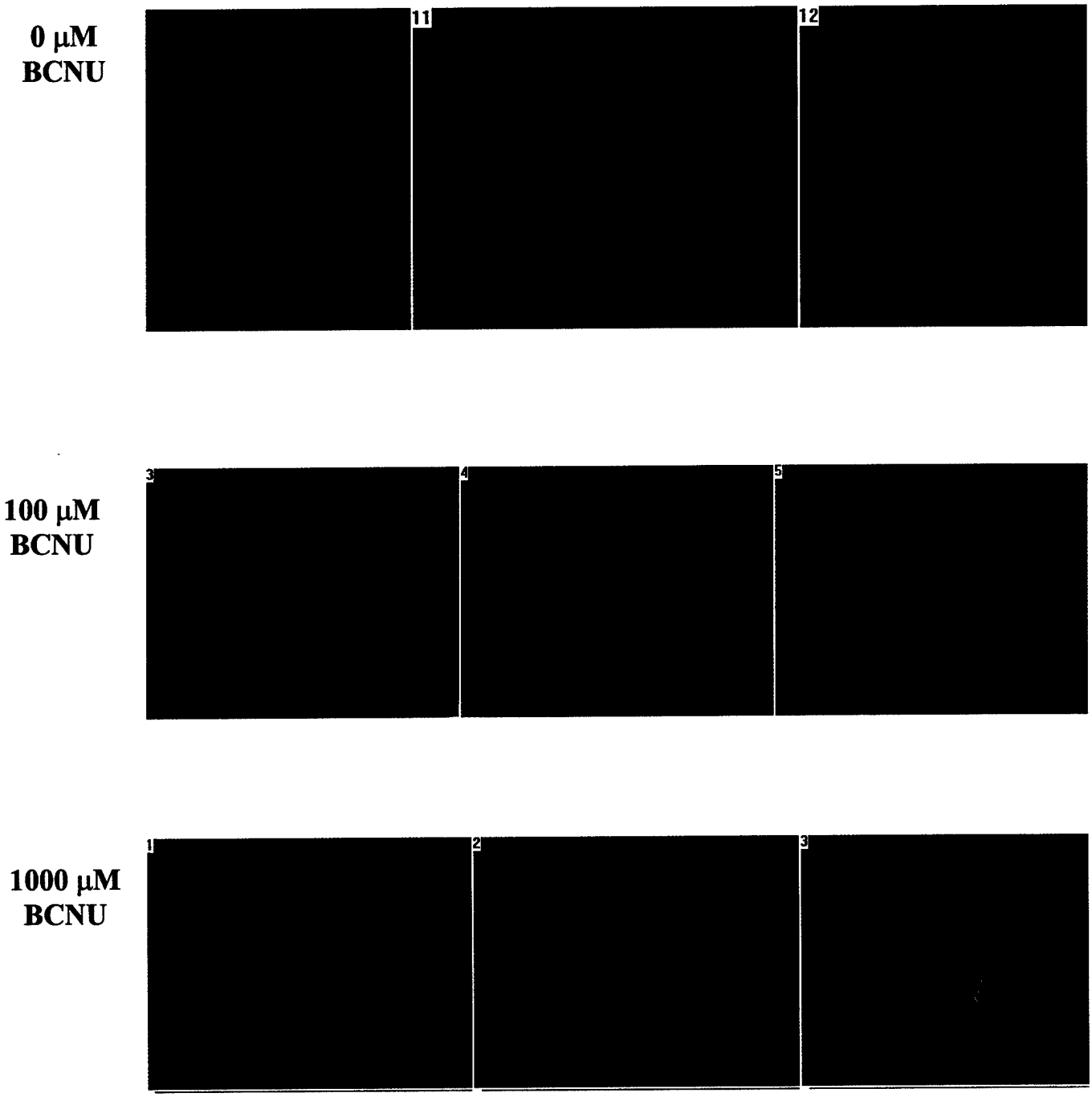
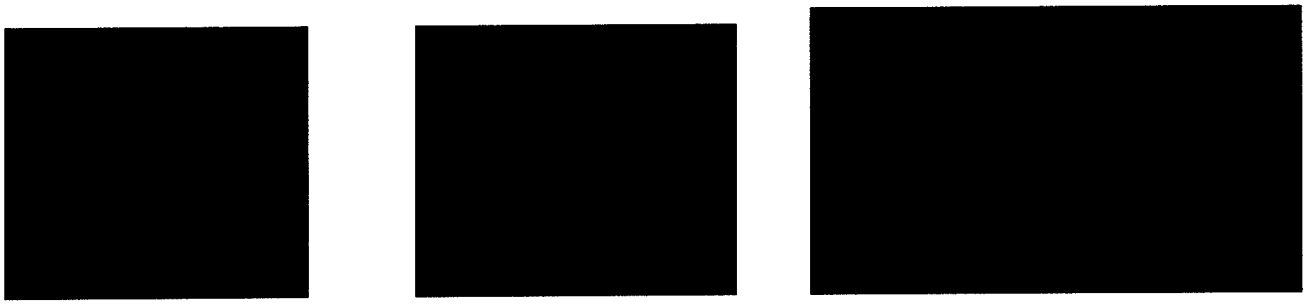


Fig. 11 Comparison of Staining After the Comet Assay With the DNA Dyes: SYBR Green and To-Pro 3

SYBR Green DNA Stain

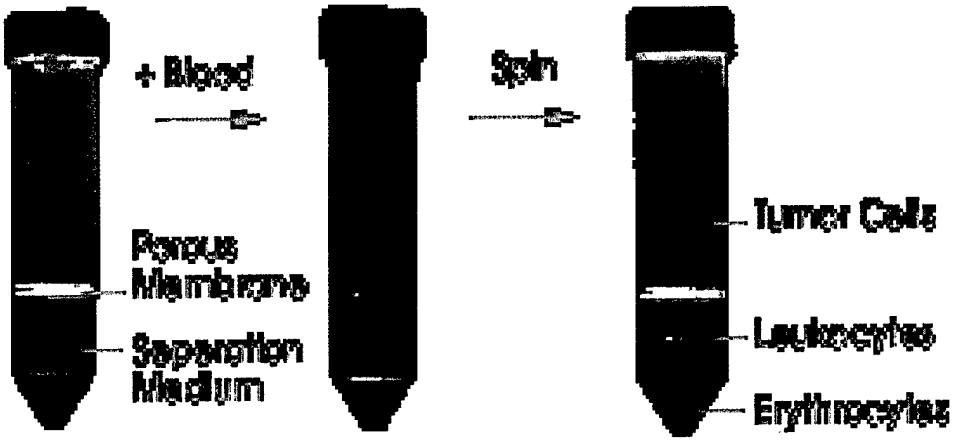


0 μM BCNU GST-pi 300 μM BCNU GST-pi 1000 μM BCNU GST-pi

To-Pro 3 DNA Stain

0 μM BCNU GST-pi 300 μM BCNU GST-pi 1000 μM BCNU GST-pi

Fig. 12 Spiking Experiment Separating MDA Breast Cancer Cells from Bone Marrow Samples Using the OncoQuick Method



(Picture is from the OncoQuick Website)

Fig. 13 Structure of Dynabeads by DynalBiotech for Immunomagnetic Cell Separation

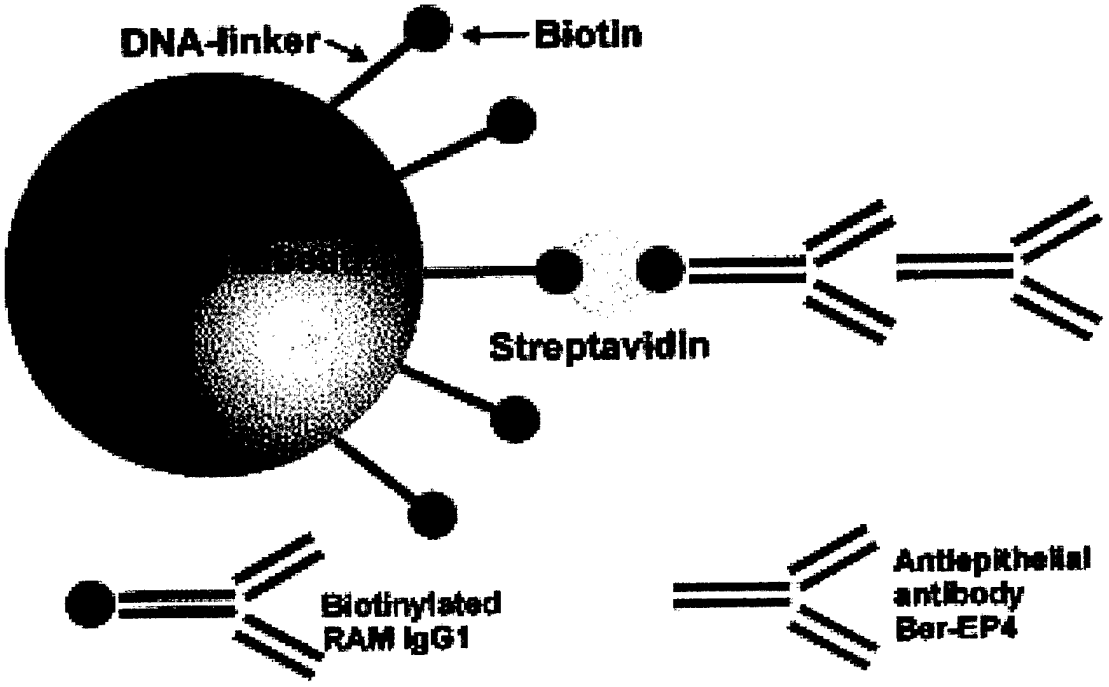


Fig. 9. The structure of Dynabeads M-280 (2.8 μ m in diameter) coated with a rat monoclonal antibody (r-Mab) against mouse IgG1. The r-Mab was biotinylated and attached to Dynabeads via streptavidin and a DNA linker. The anti-epithelial monoclonal IgG1 mouse antibody Ber-EP4 was conjugated to the bead–secondary antibody complex, and acted as the primary capture antibody. (Picture from reference 47)

Fig. 14 The Principle of Immunomagnetic Cell Separation

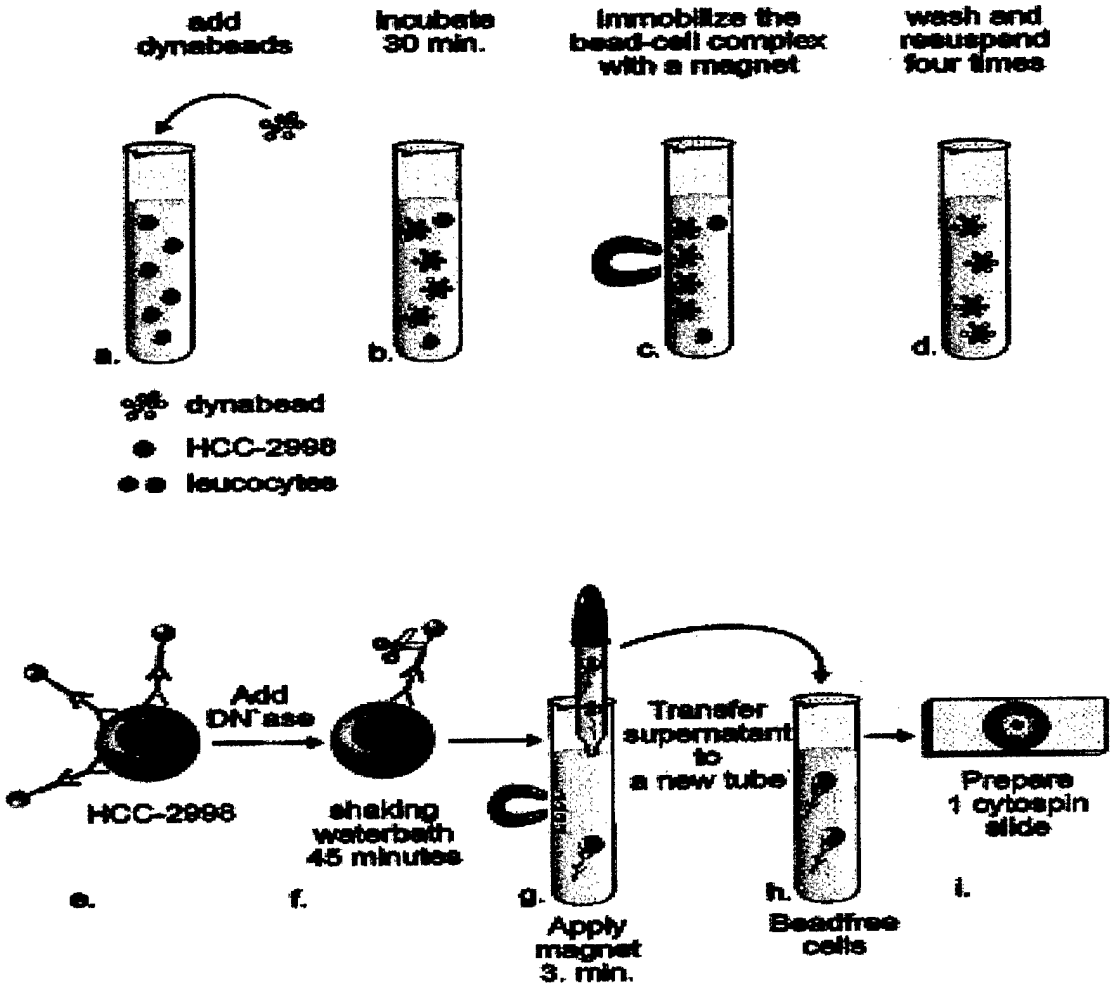
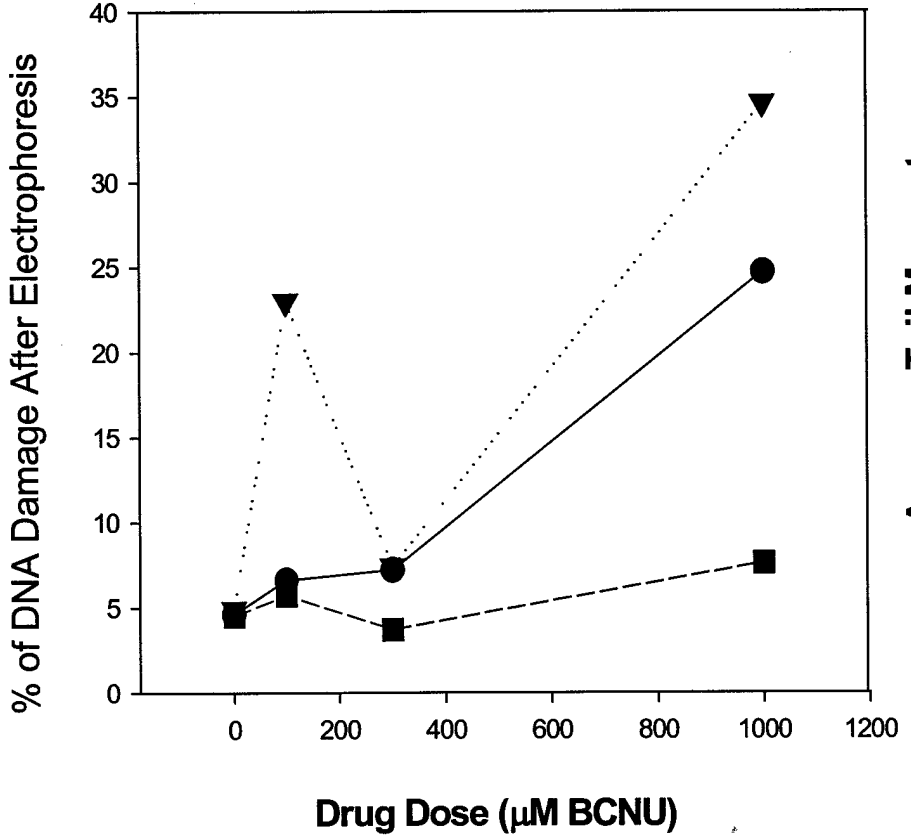
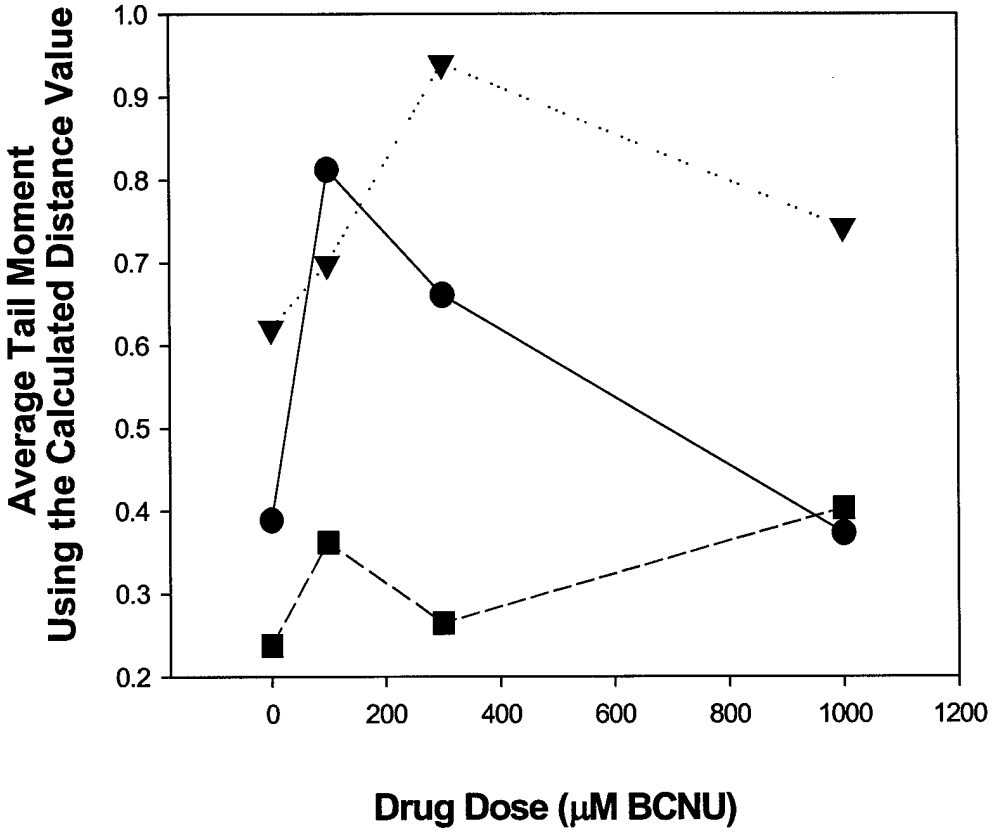


Fig. 10. Mixing of HCC-2998 cells with MNCs (a), incubation while gently tilting and rotating at 4°C (b), recovery of the carcinoma cells (surrounded by the beads as rosettes) by magnetic attraction (c), additional washes to ensure efficient removal of non-epithelial cells (d), resuspension in 200 l RPMI 10% FCS and addition of DN'ase (e), incubation on a shaking water bath at 37°C while gently mixing (f), magnetic attraction of the detached dynabeads (g), transfer of the released cells to a new test tube (h), and preparation of one cytospin slide for immunocytochemical staining (i). (Picture from reference 47)

Fig. 15

Cell Separation Spiking Experiment with MCF-7 WT and Bone Marrow Cell Mixture After Treatment with BCNU and Antibody Staining with Cytokeratin and GST-pi After Electrophoresis





MCF-7 WT: BM Mix (1:400) - After Cell Separation with Beads

MCF-7 WT Cells Only - No Beads

Bone Marrow Cells Only - No Beads

Fig. 16

Three Separate Experiments with MCF-7 WT and Bone Marrow Cell Mixtures Using the Multi-Well Trevigen CometSlides™ After Treatment with BCNU Antibody Staining with Cytokeratin and GST-pi

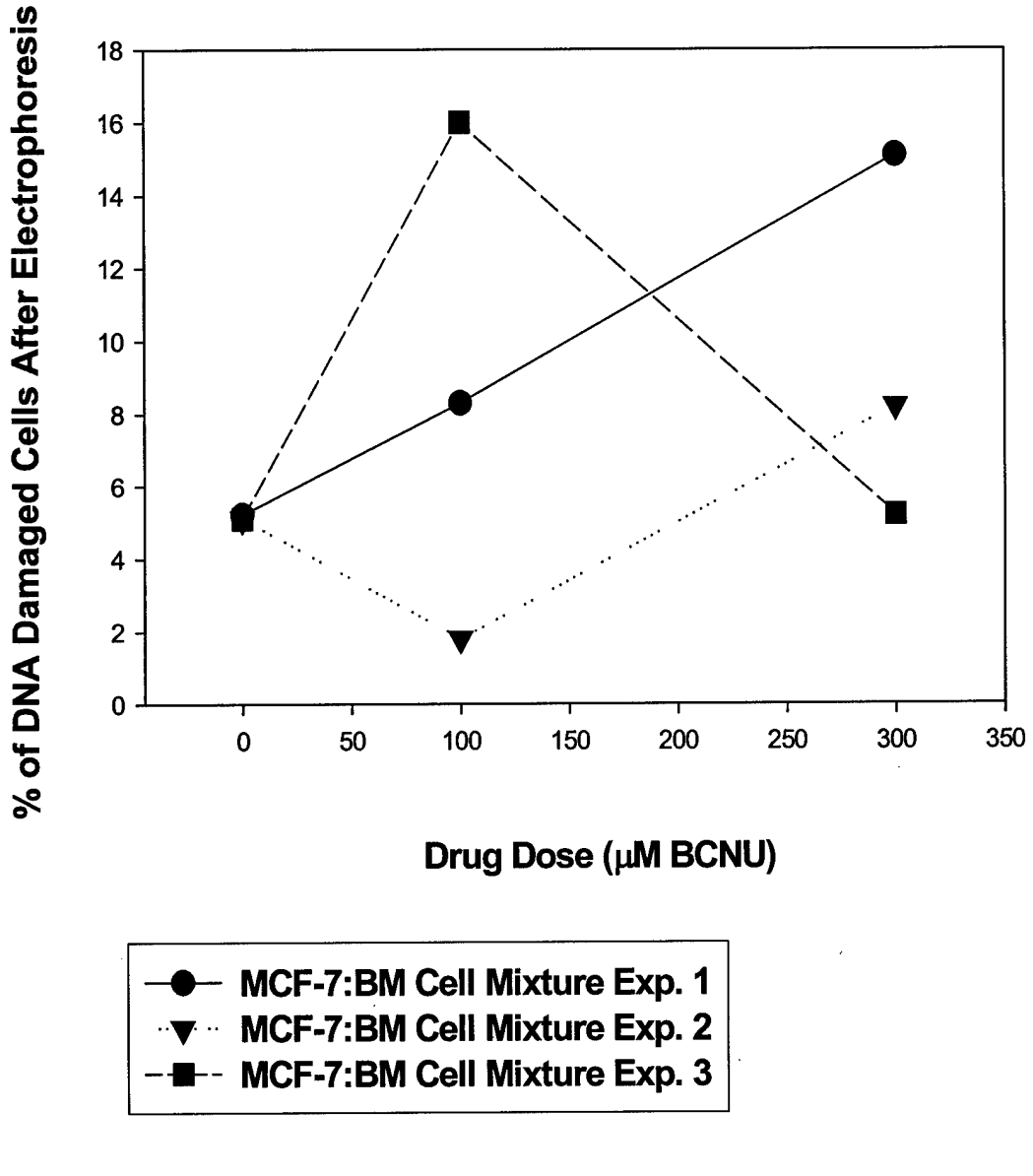
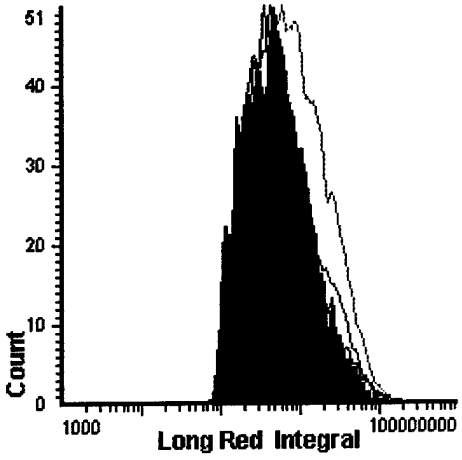


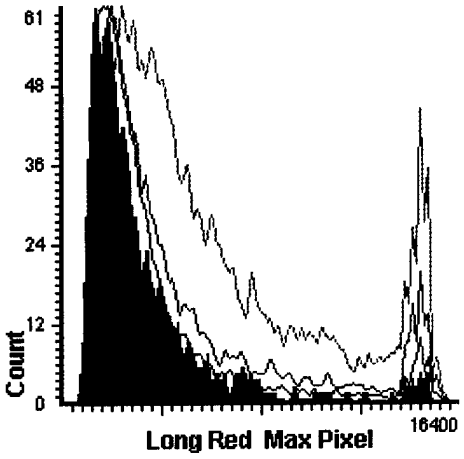
Fig. 17 Separating Out MCF-7 Cells from BM (1:1000 MCF-7:BM Cells) Using Magnetic Beads and Cytokeratin Antibody Kit

MCF-7 Cells Only

Long Red Integral Histogram



Long Red Max Pixel Histogram



For the first three drug doses we observed an increase in the max pixel values and integral intervals. At the highest drug dose these values decreased.

Black = 0 μM BCNU control

Red = 100 μM BCNU

Green = 300 μM BCNU

Blue = 1000 μM BCNU

Fig. 18 Picture of the Laser Scanning Cytometer

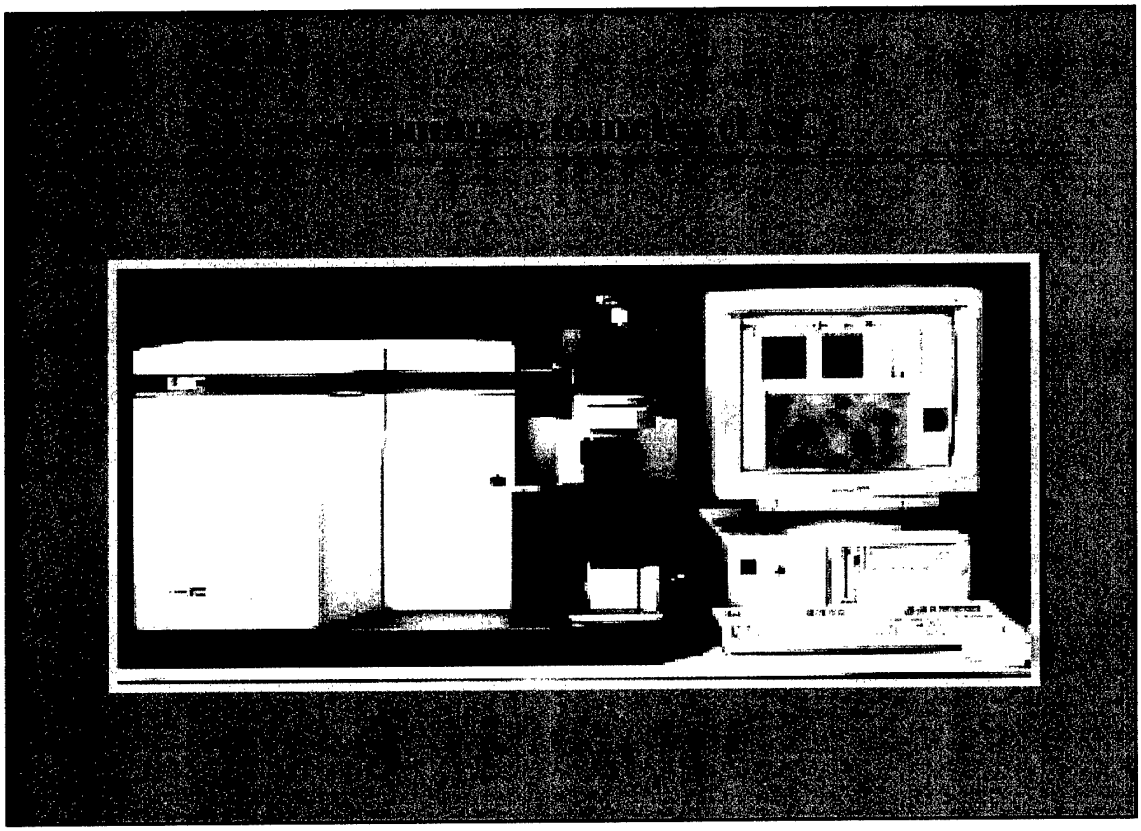


Fig 19 An Example of Scattergrams and Histograms for the Laser Scanning Cytometer for a Patient Sample

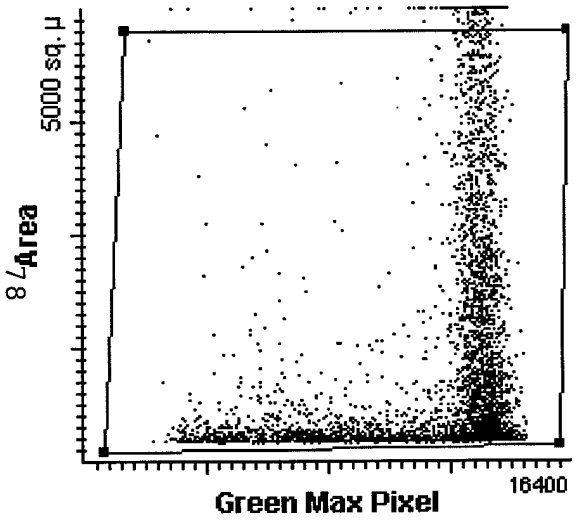


Fig. a. Scattergram. A bigger area means that there was a lot of damage. The bigger the area the bigger the comets.

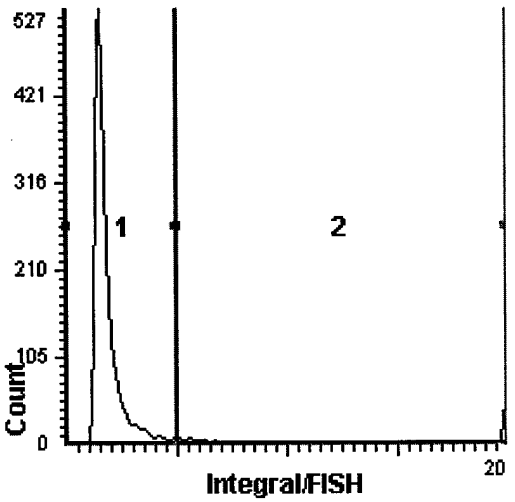


Fig. b. Histogram using ratios. This sample does not have a lot of damage because it has very low Integral/FISH value. The integral value is the measure of the whole cell, and FISH is the measure of the head. When there is a high Integral/FISH there is a lot of DNA damage.

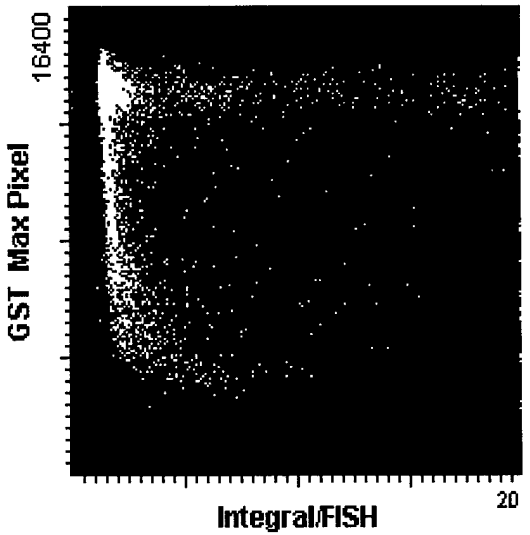


Fig. c. Scattergram using ratios. High GST value means that there is increases cellular resistance.

Fig. 20 Scheme Representing the Major Components of the Laser Scanning Cytometer

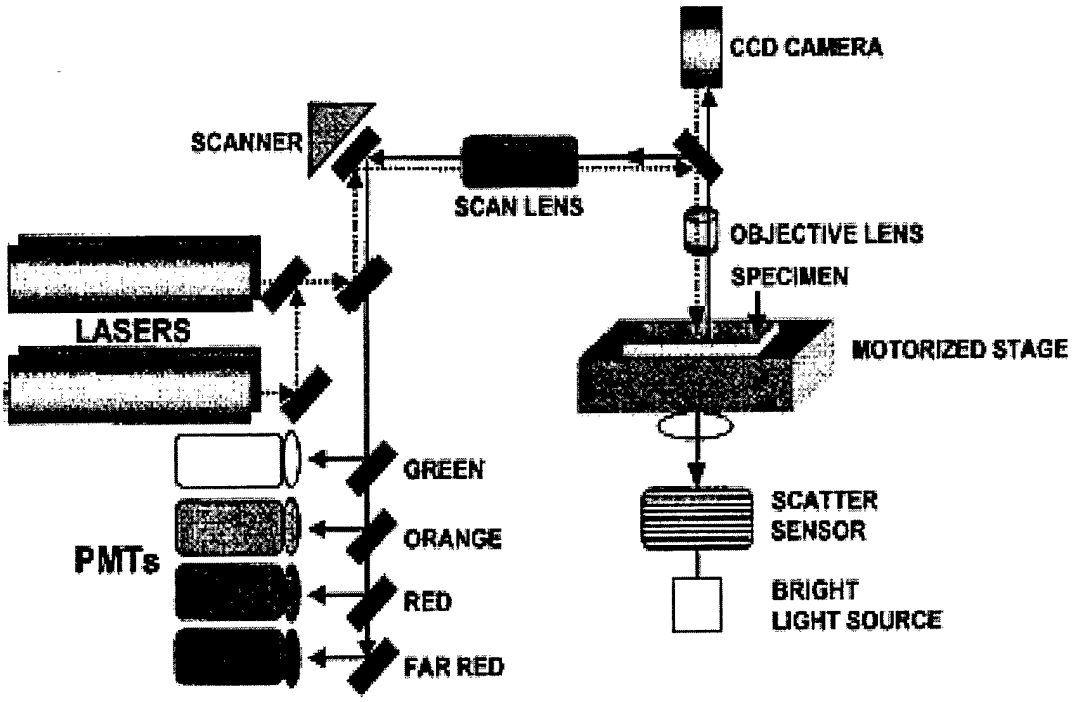
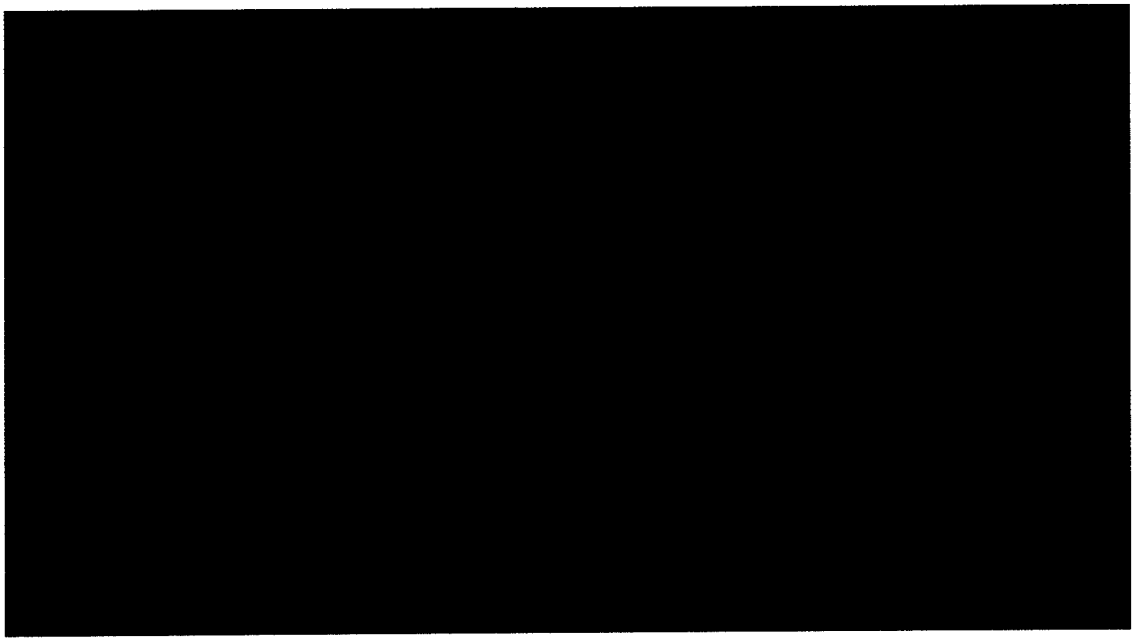
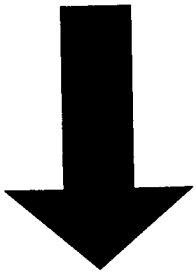


Fig. 21 Contouring Cells During The First Scan After GST Staining





Close-up of the Computer Image of a Cell with Set Contours

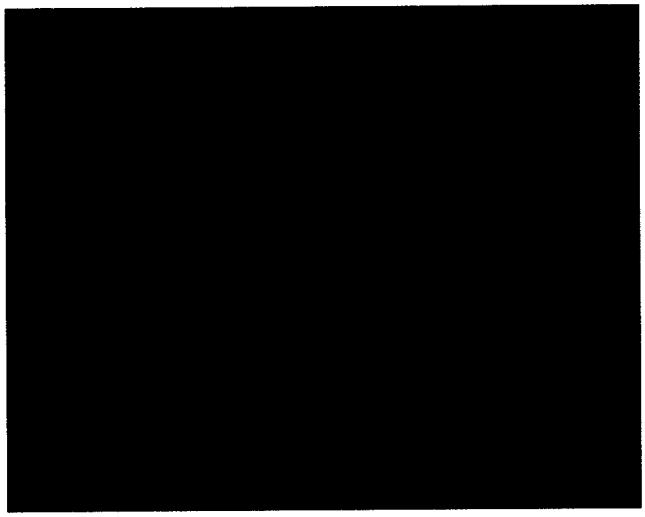
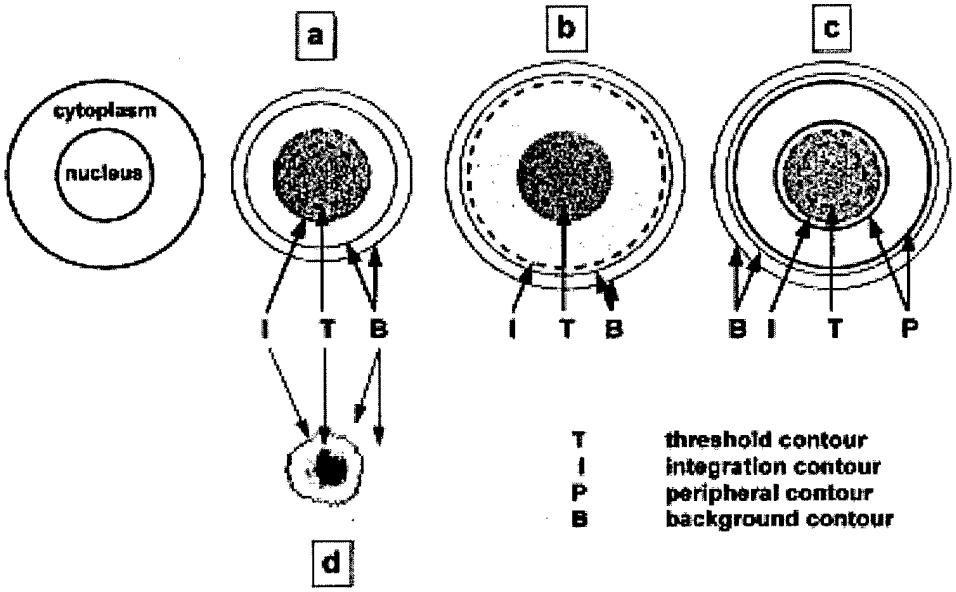
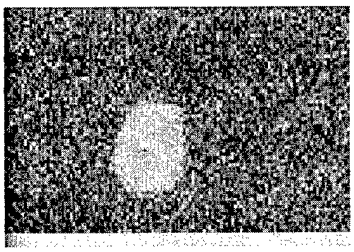


Fig. 22 Different Contour Setting for Analysis of Nuclear, Total, and/or Cytoplasmic Fluorescence Using the Laser Scanning Cytometer

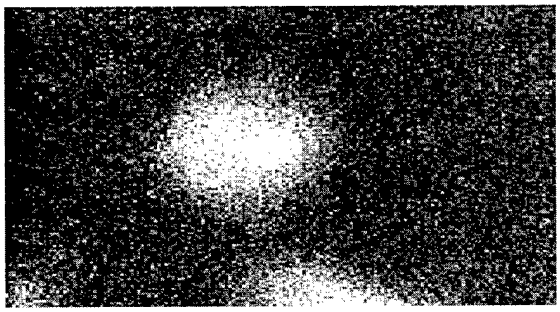


When nuclear DNA is stained with a green fluorescing dye (GST antibody), the threshold contour (T) is set on green signal to detect the nucleus, e.g. as in a. The integration contour (I) is then set a few pixels outside of T to insure that all of the nuclear fluorescence is measured and integrated (a). However, when cytoplasmic fluorescence is also measured, I is set far away from T (b). It is also possible to separately measure nuclear and cytoplamic fluorescence as shown in c by setting peripheral contours (P). In each case, the background contour is automatically set outside the cell and the background fluorescence is subtracted from nuclear, cytoplasmic, or total cell fluorescence. The actual cell contour,s as they appear in the monitor, are shown in d.

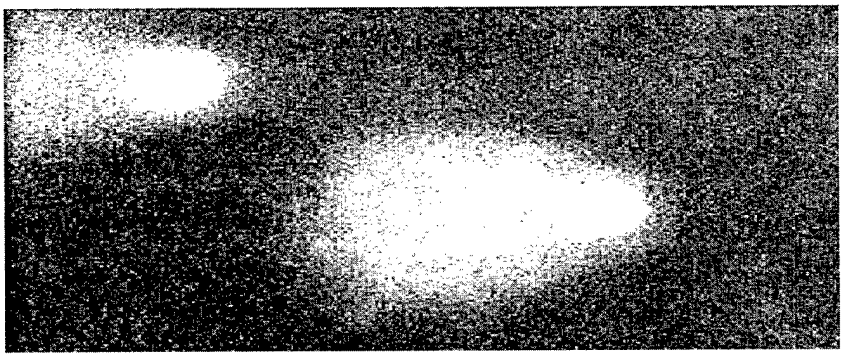
Fig. 23 Example of Pictures of Comet Images from Patient 1 Before Induction Time Sample



Pt. 1 0 μM BCNU

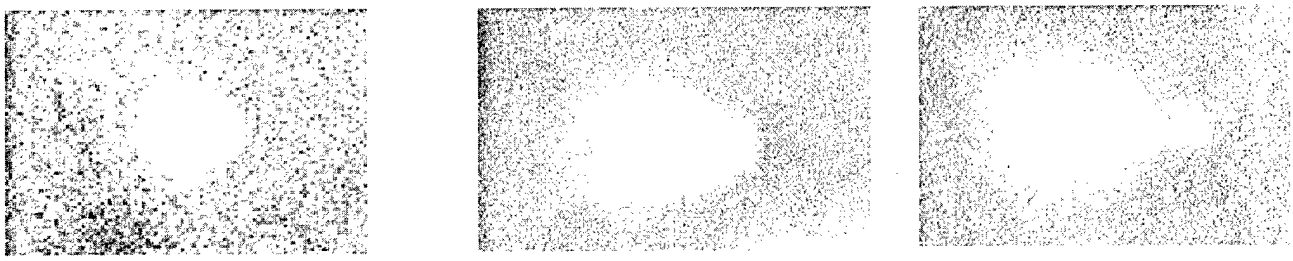


Pt. 1 100 μM BCNU

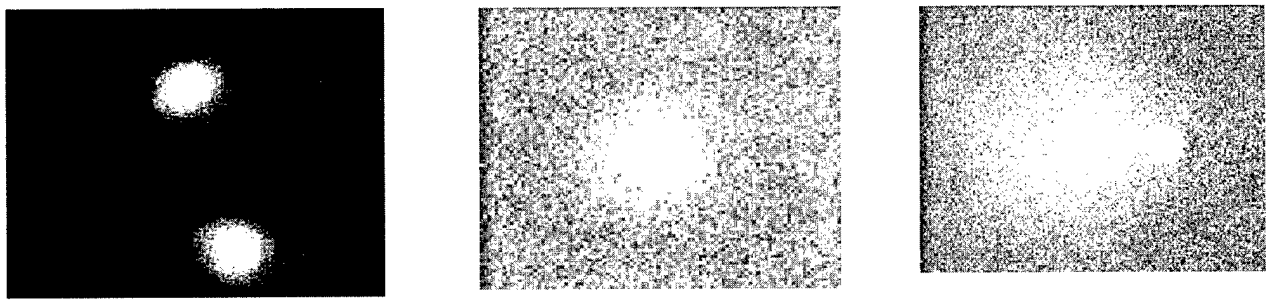


Pt. 1 1000 μM BCNU

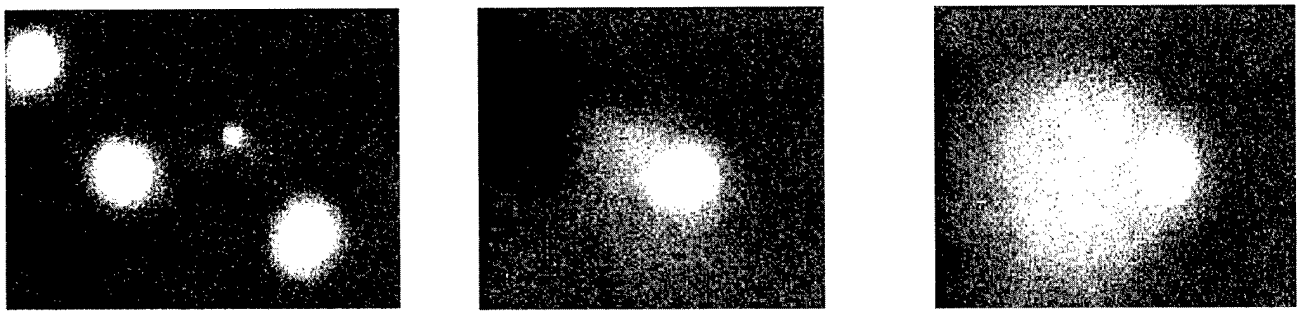
Fig. 24 Variability in Comet Size for Each Drug Dose For Patient 1



0 μM BCNU BI



100 μM BCNU BI



1000 μM BCNU BI

Fig. 25 Patient Samples without MicroMetastasis to the Bone: Data for Patient 2

Fig a. Measuring the % of Damaged Cells After Electrophoresis..

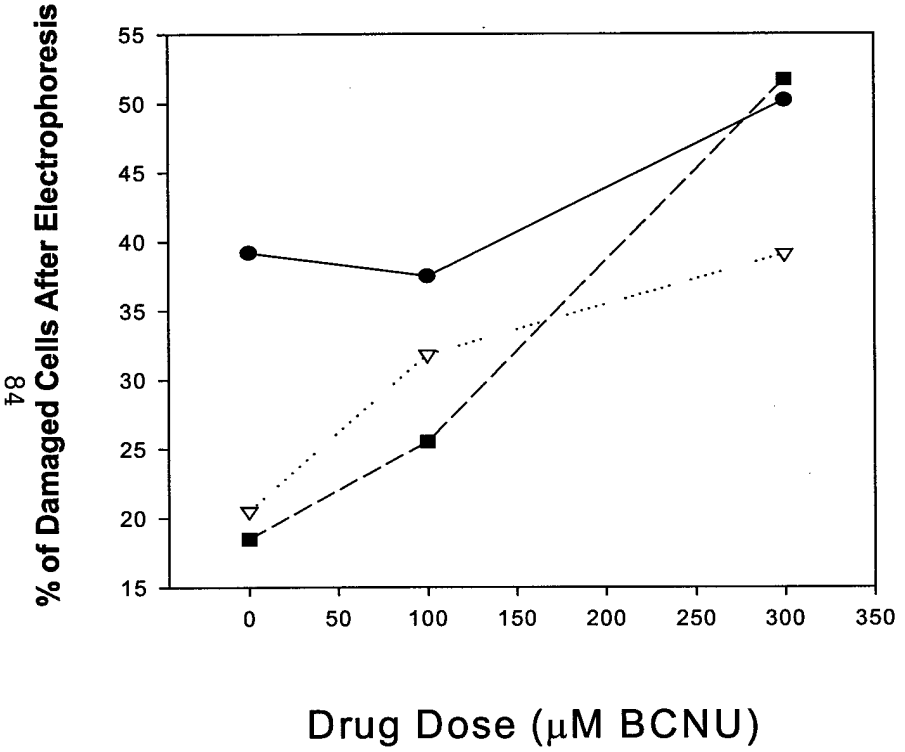
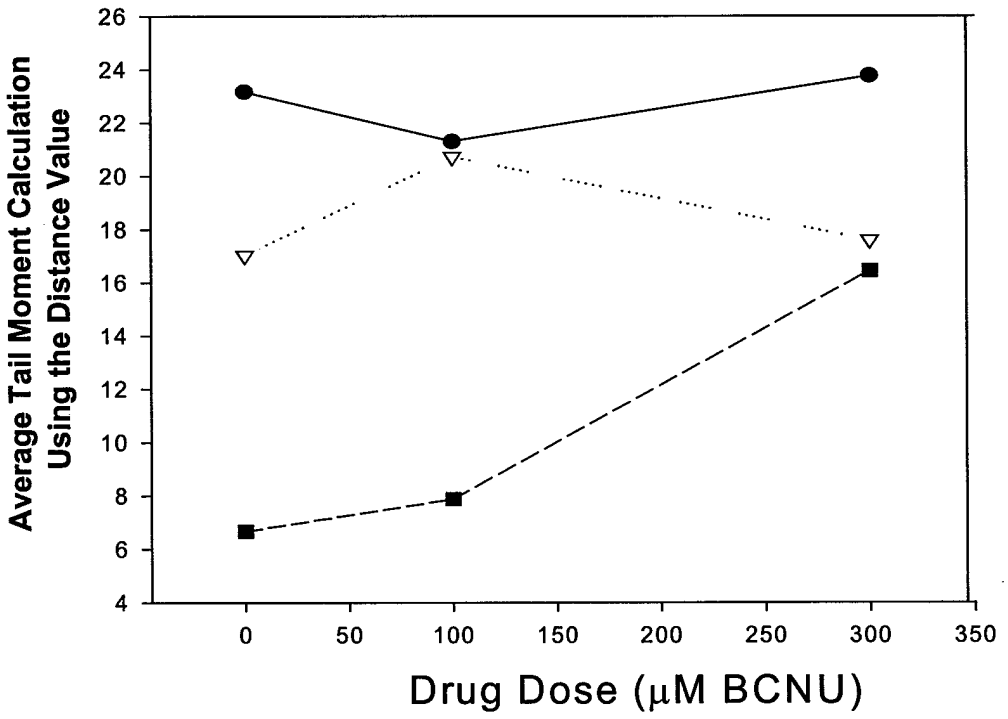


Fig b. Measuring the Average TM Using the Calculated Distance Value.



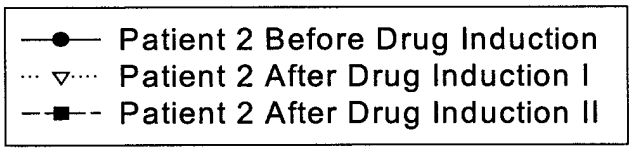


Fig. 26 Tables 1-4 Patients 1 and 2 Sample Data

Table 1 - Patient 1 – Calculating the Average Tail Moment Using the Calculated Distance Value

Drug Dose μM BCNU	Before Drug Induction	After Drug Induction I	After Drug Induction II
0	14.28	21.63	9.48
100	6.95	14.74	10.81
300	11.07	19.12	10.55
1000	8.00	13.59	8.90

Table 2 - Patient 1 – Measuring % of Damaged Cells for Dose Response

Drug Dose μM BCNU	Before Drug Induction	After Drug Induction I	After Drug Induction II
0	28.10	52.00	9.90
100	28.50	29.00	10.40
300	20.40	48.60	30.30
1000	20.60	39.70	5.90

Table 3 - Patient 2 – Calculating the Average Tail Moment Using the Calculated Distance Value

Drug Dose μM BCNU	Before Drug Induction	After Drug Induction I	After Drug Induction II
0	23.16	17.02	6.67
100	21.30	20.72	7.80
300	23.76	17.59	16.44
1000	30.05	17.91	9.18

Table 4 - Patient 2 – Measuring % of Damaged Cells for Dose Response

Drug Dose μM BCNU	Before Drug Induction	After Drug Induction I	After Drug Induction II
0	39.20	20.50	18.50
100	37.50	31.80	25.50
300	50.20	39.10	51.70
1000	49.80	64.10	11.90

Fig. 27 Comet Assay Dose Response for Two Separate Experiments Using Standard Slides After BCNU Treatment and No GST Staining MCF-7 ADR-R Comet Cells After Electrophoresis

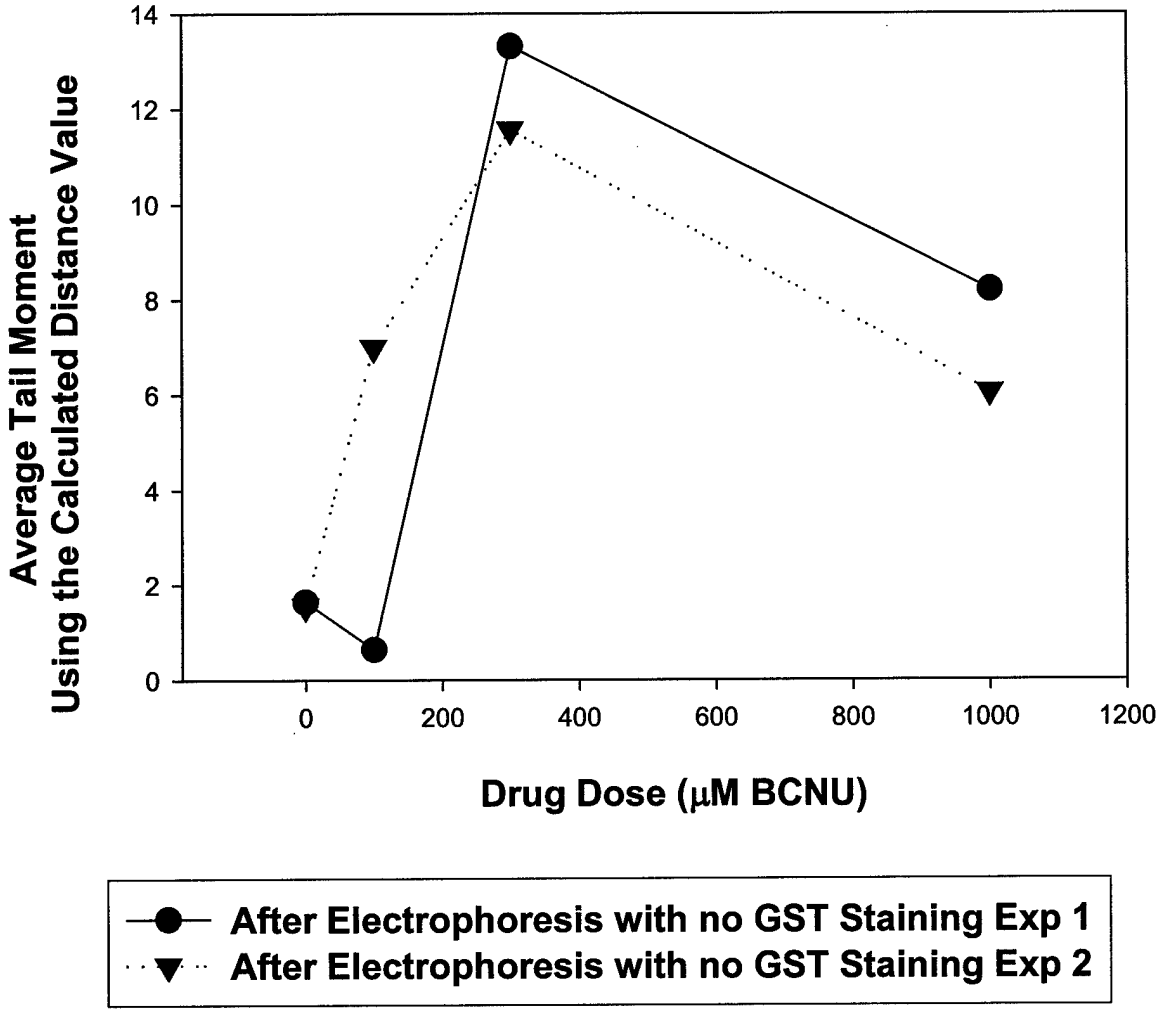


Fig. 28 Comet Assay Dose Response for Using Standard Slides After BCNU Treatment with or without GST Staining with Cadmium in First Staining Solution Only MCF-7 ADR-R Comet Cells After Electrophoresis

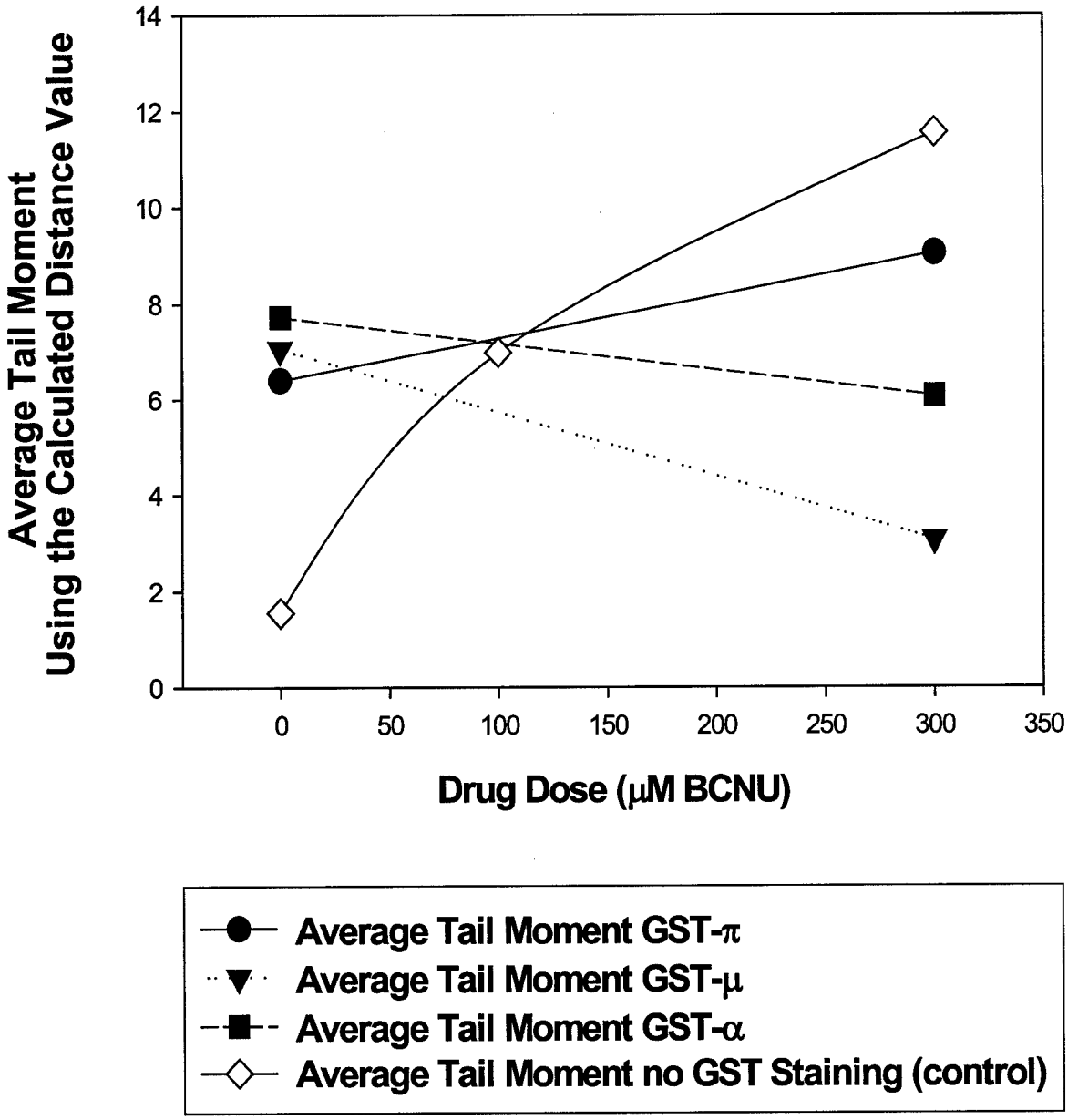
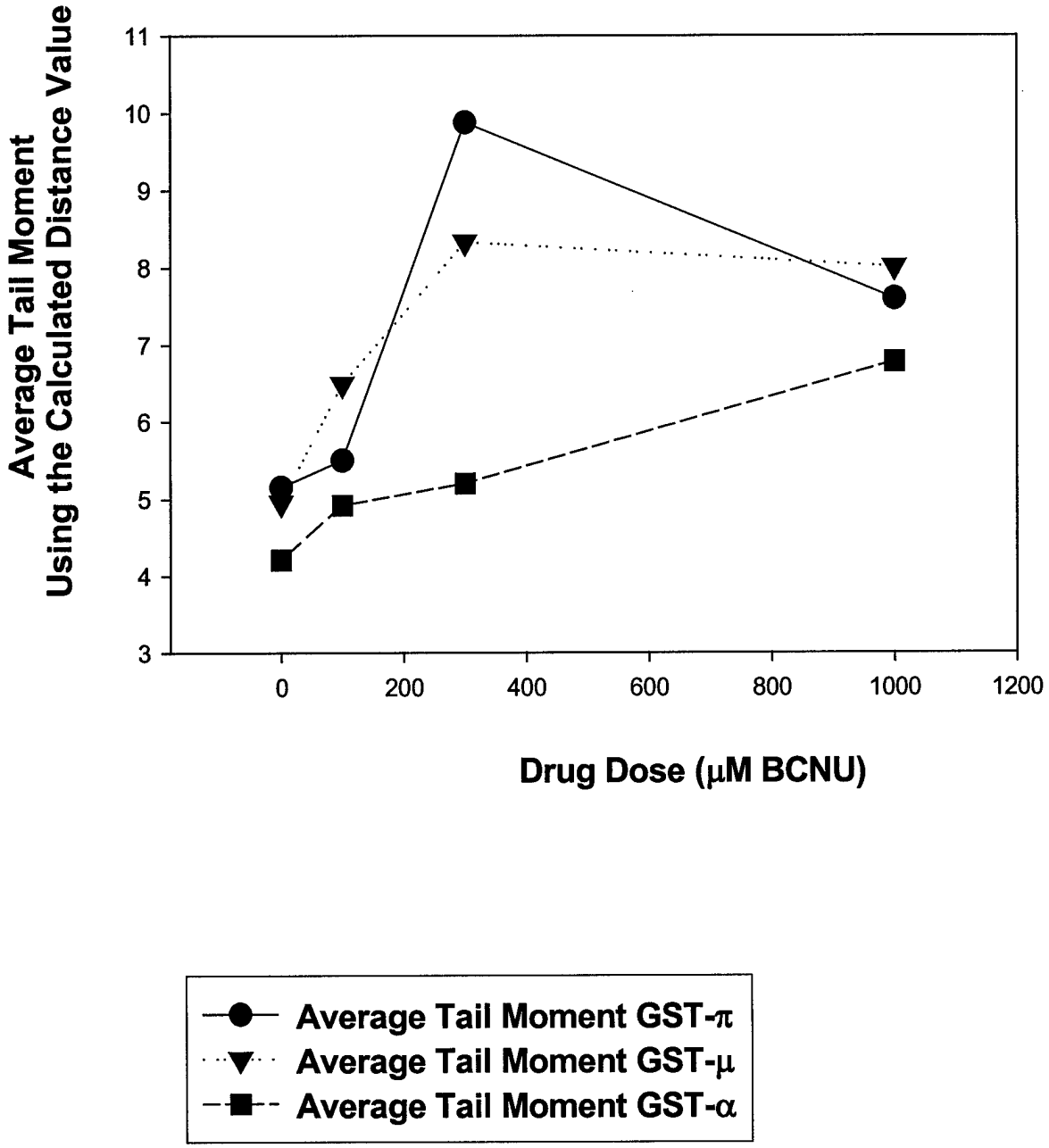


Fig. 29 Comet Assay Dose Response for Using Standard Slides After BCNU Treatment with GST Staining with Cadmium in All of the Staining Solutions
MCF-7 ADR-R Comet Cells After Electrophoresis



B. List of Standard Operating Procedures (SOPs)

1. SOPs for Older Methods for Studying DNA Damage
    a. HPLC Analysis of DNA Adducts
    b. DNA Isolation
    c. DNA Precipitation
    d. PCR Based Approaches to Adduct Analysis
    e. *In Vitro* Exposures
    f. DNA Digestion
2. Original Comet Assay Method, Instructions, and Setup Booklet
3. Trevigen CometSlide™ Comet Assay Method
4. Suggested Media Preparation of EF Media
5. Maintaining and Starting MCF-7 WT and Adr-R Cell Lines
6. The OncoQuick Cell Separation Method
7. Dynal Bead Separation – Attaching and Removing the Beads
8. Ficoll Cell Separation Method with Patient Samples
9. All of the Combined Methods Using the Standard Comet Method with Smaller Areas
    a. Equipment and Materials
    b. Preparations Before Experiment
        i. Conjugating the Antibody
        ii. Making up Buffers and Solutions
        iii. Comet Assay Supplies, Solutions, and Preparations
    c. Preparation of MCF-7 cells or Bone Marrow Samples
    d. Procedure for Determining Drug Dosage – Setting up Dilutions
    e. Drug Treatment and Incubation
    f. Immunomagnetic Cell Separation with Beads Attached and Removed from Tumor Cells
    g. Immunocytochemistry – GST and Cytokeratin Antibody Staining
    h. The Comet Assay
    i. Data Analysis using WinCyte Software, Microsoft Excel, and Microsoft Access

SOP for DNA Isolation

Protocol for the Isolation of Genomic DNA

Involving Sample Preparation and Lysis for the Genomic tip Protocol

This two part protocol is designed for the preparation of up to 20ug of genomic DNA from up to 5 x 10^6 of cultured or mononuclear blood cells from human blood or bone marrow specimens. The purified genomic DNA ranges from 20 to 150 kb in size.

The cultured cells were grown in cell suspension, and processed according to the methods described in the QIAGEN Genomic DNA Handbook Tissue Culture and Harvesting Protocol (09/97).

Part I: Sample & Reagent Preparation and Lysis Protocol for Cultured Cells

Procedure as follows

Sample Preparation:

Suspension cultures of lymphocyte lines typically contain 2x10 7^{th} cells /ml. 20 G tips will isolate DNA from 5 x 10 6^{th} cells, so use 0.4 ml per tip max, or an equivalent number of human cells.

Us a 15ml point bottom centrifuge tube – Corning #_____or Falcon #_____

Isolation of genomic DNA from cell suspension of cultured cells:

- ☐ **1. Centrifuge the appropriate number of cells (5 x 10^6) for 10 min. @ 1500 x *g* (@ 4*C) in a 15 ml centrifuge tube.**
- ☐ **2. Discard the supernatant, ensuring all media is completely removed.**
- ☐ **3. First Wash cells in 4ml of PBS (same centrifuge conditions)**
- ☐ **4. Discard the supernatant, ensuring all media is completely removed.**
- ☐ **5. Second Wash cells in 4ml of PBS (same centrifuge conditions)**
- ☐ **6. Discard the supernatant, ensuring all media is completely removed.**
- ☐ **7. Resuspend in 0.5 ml of PBS (4*C) to a final concentration of ~10^7 cells/ml.**

Reagent Preparation:

- ☐ **1. To obtain maximum purity, and optimal flow rates, it is very important not to use more than 0.5 ml of this suspension.**
- ☐ **2. Store or equilibrate Buffer C1 and distilled water in a 50 ml beaker (autoclaved) @ 4*C in refrigerator.**

❑ 3. Buffers G2, QBT, QC, and QF, are equilibrated to room temperature (Recommend storing at 4 deg C).

{ NB: Buffer C1 and distilled water must be kept on ice during procedure. }

Lysis Protocol for Cultured Cells grown in a Cell Suspension

❑ 1. To a 14 ml centrifuge tube add the following:

- ❑ 1.1 One Part (e.g., 0.5 ml) from the cell suspension of Cultured Cells
- ❑ 2.2 One Part (0.5 ml) of ice cold C1 buffer
- ❑ 1.3. Three Parts (1.5 ml) of ice cold distilled water

❑ 2. Mix by inverting tube several times until suspension becomes completely translucent. Apparently however, previously frozen samples do not visibly change upon lysis.

❑ 3. Incubate on ice for 10 minutes.

❑ 4. Centrifuge the lysed cells @ 4*C for 15 minutes at 228 x g = 1000 rpm. Note: 1300 g is recommended in the QIAGEN protocol, which is ~ 2300 rpm. We found this rapid centrifugation may cause the cells to clump and prevent them from complete lysis and protein denaturization. May vary with the type of cells being extracted. If using buffy coats pelleted nuclei is still red after centrifugation, this is due to residual hemoglobin, may need to repeat this step)

❑ 5. Discard the Supernant and Add:

- ❑ 5.1. 0.25ml of ice-cold C1 buffer
- ❑ 5.2. 0.75ml of ice-cold distilled water to sample.

❑ 6. Resuspend the pelleted nuclei by vortexing.

❑ 7. Centrifuge 228 x g (1000 rpm) for 10 min., and discard supernatant. (May Freeze Pellets at this point, using cryo-microfugetube)

❑ 8. Start and set the water bath @ 50*C. Use oscillating Blue Water Bath.

❑ 9. Add 1ml of G2 buffer and completely resuspend the nuclei for 1min. Resuspend nuclei as thoroughly as possible by vortexing as this step is critical a good flow rate on the Genomic tip.

❑ 10. Add 35.7ul.of preprepared Proteinase K and incubate @ 50*C for ~50 minutes. Note: the QIAGEN protocol uses 25ul; I increased this amount to make the

necessary adjustments per grammage needed Also, the length of incubation depends on how well the nuclei were resuspended in the last step.

Part II: Genomic-tip Protocol

- ☐ 1. Equilibrate a QIAGEN Genomic tip 20/G with 1ml. of QBT buffer and allow to empty by gravity flow.
- ☐ 2. Vortex sample for 10 seconds and add to the equilibrated Genomic tip PROMPTLY.
- ☐ 3. Wash the QIAGEN Genomic tip with 1ml of QC buffer: 3 times.
- ☐ 4. Elute the genomic DNA with 1ml. of QF buffer: 2 times with new collection tubes to collect the eluate.
- ☐ 5. Precipitate the DNA by adding 1.4ml. of isopropanol (@room temp.) To the eluted DNA.
- ☐ 6. Precipitate the DNA by immediately centrifuging @ ~>5000 x g (~8500 rpm) for 15 minutes @ 4*C. Carefully remove supernatant.
- ☐ 7. Wash the centrifuged DNA pellet with 1ml. of cold 70% ethanol.
    - o Vortex briefly and centrifuge @ ~>5000 x g (~8500 rpm) for 10 minutes @ 4*C.
    - o Carefully remove the supernatant without disturbing the pellet.
    - o Air-dry for 5-10 minutes and resuspend the DNA in 0.1-2ml. of T.E. buffer (pH 8)
- ☐ 8. Dissolve the DNA overnight on a shaker or @ 55*C for 1 - 2 hours.

PRECIPITATION OF DNA WITH ETHANOL

1 Adjust the concentration of the purified DNA, by adding 10M ammonium acetate to make a final solution that contains 2M ammonium acetate. This is done by estimating the volume of the DNA solution that is in TE buffer (pH 8.0). This is necessary to facilitate precipitation of DNA by ethanol.
2 Add twice as much of cold 100% ethanol as the entire volume of the sample.
3 Mix and store on ice for 10 minutes. (If the DNA is smaller than 1 kb or present at a concentration less than 100 ng/mL, the solution should be stored at -70 degrees Celsius for about 4 hours. For DNA less than 0.2 kb in size, the addition of 0.1 M $MgCl_2$ improves recovery.)
4 Centrifuge at 4 degrees Celsius for 10 minutes in a microcentrifuge at top speed (14, 000 rpm).
5 Discard the supernatant.
6 Invert the tubes on a layer of absorbent paper in the hood for an hour, to allow drainage of ethanol. Solvent traces can be removed in a vacuum desiccator or vacuum centrifuge.
7 Dissolve the pellet in 25 uL of HPLC grade water (pH 8.0). Rinse the tube walls with water to ensure dissolution of DNA. Heating to 37 degrees Celsius for at least 5 minutes may help solubilize the DNA as well. Store the DNA solution at 4 degrees Celsius for digestion of DNA.

Conversion of the final solution to 0.2 M

$C_1V_1 = C_2V_2$

C_1 = Conc. Of ammonium acetate
V_1 = Volume of ammonium to add
C_2 = Conc. Of final solution
V_2 = Volume of final solution

Conversion of DNA to make the solution 0.2 M

$C_1V_1 = C_2V_2$

C_1 =	10 mol / 1000 mL	C_1 = 0.01 M
V_1 =	x mL (x uL)	V_1 = 0.25 mL
C_2 =	2 mol / 1000 mL	C_2 = 0 M
V_2 =	(1mL + x mL)	V_2 = 1.25 mL
x =	?	x = 0.25 mL

Reagent	Amount (uL)
Sample	1000
A. Acetate	250
Ethanol	2500
HPLC H_2O	25

Reagents should be dated with the date they are prepared.
Kit components with the date they were opened.
Dates should be added to experiments.

	REAGENTS USED:					
		Date		Lot #	Place of Storage	
	dNTP Blend	10/31/01		36227311017	Freezer A	
	10X SYBR PCR Buffer	10/31/01		430488911011	Freezer A	
	25mM MgCl2	10/31/01		430489811018	Freezer A	
	AmpliTaq Gold	10/31/01		A02808	Freezer A	
	AmpErase UNG	10/31/01		430490311011		
	1st set of primers					
	NRAS A F	7/19/00				
	NRAS B R 2	7/25/00				
	2nd set of primers					
	NRAS A F	7/19/00				
	Step2 1st primer	7/19/00				
	100 bp ladder				Freezer A	Gibco
	SPECIMENS*:					
		Source		Date Prep.	Place of Storage	System
		Source		Date Prep.	Place of Storage	
1st primer	1 Sample 1	cell			-80 REVCO - C	
	2 Sample 2	cell			-80 REVCO - C	
	3 Sample 3	cell			-80 REVCO - C	
	4 Sample 4	cell			-80 REVCO - C	
	5 Sample 5	cell				
	6 Sample 6	cell				
2nd primer	7 Sample 7	cell			-80 REVCO - C	
	8 Sample 8	cell			-80 REVCO - C	
	9 Sample 9	cell			-80 REVCO - C	
	10 Sample 10	cell				
	11 Sample 11	cell				
	12 100 bp ladder					

NRAS A and NRAS B 2 & NRAS A and Step2 1st primer

# of reactions

7	Vol. Total Mix	Components	volume per reaction
Check Off	ul	**MIX 1**	**25**
	17.5	ul 10X SYBR PCR buffer - PE	2.5
	17.5	ul 25 mM MgCl2 - PE	2.5
	14	dNTP Blend	2
	1.75	NRAS A F	0.25
	1.75	NRAS B R 2	0.25
	0.875	Amplitaq Gold polymerase From PE	0.125
	1.75	AmpErase UNG	0.25
	105.88	water	15.125
Sum	161	Volume Mix Per Tube ul	23
		Check on vol total mix	161

6	Vol. Total Mix	Components	volume per reaction
Check Off	ul	**MIX 2**	**25**
	15	ul 10X SYBR PCR buffer - PE	2.5
	15	ul 25 mM MgCl2 - PE	2.5
	12	dNTP Blend	2
	1.5	NRAS A F	0.25
	1.5	Step2 1st primer	0.25
	0.75	Amplitaq Gold polymerase From PE	0.125
	1.5	AmpErase UNG	0.25
	90.75	water	15.125
Sum	138	Volume Mix Per Tube ul	23
		Check on vol total mix	138

Suggest: Add water first, then PCR mix, then sample.

Tube Number	Specimen	sample vol ul	Vol PCR Mix ul	Total Vol Calc	Total Vol Planned
1	Sample 1	2 **MIX 1**	23	25	25
2	Sample 2	2 **MIX 1**	23	25	25
3	Sample 3	2 **MIX 1**	23	25	25
4	Sample 4	2 **MIX 1**	23	25	25
5	Sample 5	2 **MIX 1**	23	25	25
6	Sample 6	2 **MIX 1**	23	25	25
7	Sample 7	2 **MIX 2**	23	25	25
8	Sample 8	2 **MIX 2**	23	25	25
9	Sample 9	2 **MIX 2**	23	25	25
10	Sample 10	2 **MIX 2**	23	25	25
11	Sample 11	2 **MIX 2**	23	25	25
12	100 bp ladder				

Running EZ-Gel

Label GeneAmp tubes to match samples
Use Sigma Gel Loading Solution
Carefully press start once for 2 min prerun; Then load specimens, then press a sceond time for Run. Time Run

	Tube	DEPC H20 ul	Loading Soln	Sample ul	
Sample 1	1	16	0	4	Place in a 65 deg C MJ unit for 10 min and place
Sample 2	2	16	0	4	immediately on ice.
Sample 3	3	16	0	4	
Sample 4	4	16	0	4	
Sample 5	5	16	0	4	
Sample 6	6	16	0	4	
Sample 7	7	16	0	4	
Sample 8	8	16	0	4	SAVE TUBE 12 in FREEZER
Sample 9	9	16	0	4	
Sample 10	10	16	0	4	
Sample 11	11	16	0	4	
100 bp ladder	**CONTROL**	19	0	1	PLACE IN A WATERBATH FOR 5 MIN (37*C) and put immediately on ice

Time Started: **Time Stopped:**

NRAS A and NRAS B PCR Mix using Taq polymerase

Sample	temp. vol	abbrev. Id.	sample type	extr. on	results
1	2	Sample 1	cell		
2	2	Sample 2	cell		
3	2	Sample 3	cell		
4	2	Sample 4	cell		
5	2	Sample 5	cell		
6	2	Sample 6	cell		
7	2	Sample 7	cell		
8	2	Sample 8	cell		
9	2	Sample 9	cell		
10	2	Sample 10	cell		
11	2	Sample 11	cell		
12	2	100 bp ladder			

Cells: Date of Preparation:

Recovery Standard:
Salmon Testes DNA in 0.1 M Tris Buffer pH 7.2 2/2/00
- See setup for DNA digestion experiments

10 M Ammonium Acetate Buffer

ammonium acetate (NH_4COOCH_3)

MW = 77.08 g/mol

Molarity (mol/L)	MW (g/mol)	Amt (g/L)
10	77.08	770.8

Modifications to only make 500 mL of buffer instead of 1L

Amt (g/L)	Conversion factor (L/mL)	New amt (g/L)
770.8	0.5/500	384.4

CELL COUNTS

Cell count is determined by Trypan blue exclusion by the following:

62.5 ul Trypan blue
37.5 ul Hank's balanced salt solution
25 ul cell suspension
mix and let stand for 5 min. and count on a hemocytometer
take avg. per square multiplied by 5x10e4

cell count = 180,000

The following chart will make the calculations. Enter the values for each square counted.

sample	A		B		C	
square	live	dead	live	dead	live	dead
1	4	0	5	1	0	0
2	0	0	1	0	0	0
3	5	1	1	0	0	0
4	0	0	2	0	0	0
5	3	1	0	0	0	0
6	5	0	2	0	0	0
7	2	1	2	0	0	5
8	3	0	4	1	0	0
9	7	0	1	0	0	0
10	8	1	3	0	0	0
total	37	4	21	2	0	0
divide by 10	3.7	0.4	2.1	0.2	0	0
multiply by 5	18.5	2	10.5	1	0	0
e104	185000	20000	105000	10000	0	0
Total Cells		205000		115000		0
Viability		90.2439		91.30435	#DIV/0!	#REF!

Note the changes in the volume of the cell suspension and the medium.
By changing the number of slides in the formula on this sheet, the program will automatically adjust the volumes.

This sheet was taken from this file: **C:\ann's comets\supplies2.xls** May be a later vesion of the files.

BCNU WEIGHING PROCEDURES

The following is a procedure for determining Treatment dosage Using Mettler H54AR

1. Weigh microtubes (Sarastedt 1.5 ml. microtube)
2. Adjust coarse adjustment weight using "1/2"
3. Fine tune on "1" (bars between teeth)
4. Remove static (when necessary) using static gun
5. Take microtubes to chemical exhaust hood
6. Using a spatula take and add small amount to respective tube — begin weight of tube = 1.548 g
7. Close tube and weigh on Mettler; weigh on "1" — end weight of tube w/BCNU = 1.5598 g
8. Calculate the number of Moles in the tube — weight of BCNU = 11.8 mg

To make a 1 M Standard, use formula: weight in g/Molecular Weight = ml solvent needed for 1M

Weighing

amt of (mg) =		11.8

Date of preparation: 2-9-00

Compound	MW (g/mol)	Amt (mg)	1/MW (mol/g)	1/Molarity (uL/mol)	Amt (uL)		Stock Label
BCNU	214.1	11.8	0.004670715	10000000	551.144325	= 100 mM	BCNU 100mM

101

Dilutions

Stock Label	Stock mM	Stock ul	Solvent	Solvent ul	New Conc (mM)	New Label	100 Fold Dil Gives uM
BCNU 100mM	100	100	ethanol	900	10	BCNU 10mM	100

The stds were frozen and placed in the -80 freezer

BCNU Dose Response Assay Experiment For DNA And Cultured LymphocytesCells

Cells	BCNU Conc (mM)	Tube #	mL cells	mL RPMI	mL DNA*	mL Tris	Rx (10ul)	Vol Check (mL)	Exposure / Label	Ammonium Acetate (uL)	Ethanol (uL)
	0	1	0.666	0.324			ETHANOL	1	2 hours	NA	NA
	10	2	0.666	0.324			BCNU 100mM	1	2 hours	NA	NA
	100	3	0.666	0.324			BCNU 10mM	1	2 hours	NA	NA
	0	4			0.01	0.98	ETHANOL	1	2 hours		
	10	5			0.01	0.98	BCNU 100mM	1	2 hours		
	100	6			0.01	0.98	BCNU 10mM	1	2 hours		

**Cells were processed by: SOP for DNA Isolation. This can be found at
C:Anne\DNA Adducts\Singer Bodell Method\SOP1

DRUG CALC

Calculations for treatment solution: an example with Carmustine

Treatment Molarity Values: 10, 50, 100, 500, 1000 uM

Mix one ml of the highest concentration treatment and dilute it down to provide the other concentrations.

Treatment Molarity	Molarity of stock	moles/liter X grams/mole equals mg/liter		
		moles/liter	grams/mole	equal mg/L
100 uM	10000 uM	0.01	214.1	2.14

C:\anne\DNA Adducts\ SOP FOR Cell Exposure Assays2

TREATMENT OF CELLS WITH CARMUSTINE

Molecular formula of Carmustine: $C_5H_9Cl_2N_3O_2$

Carmustine solution - a powder; mw = 214.1 g/mol
(214.1 g x 1 mole x 100umole) / (1 mole x 100,000 umole x 1000 mL)
= 0.0002141 g/ mL DMSO
This is a very small amount to weigh out (in fact impossible). Therefore, first weigh out a very small amount of drug and then next calculate its molarity.

amt of Carmustine (g) =	0.01

Compound	MW (g/mol	Amt (g)	1/MW (mol/g)	1/Molarity (uL/mol)	Amt (uL)	
BCNU	214.1	0.01	0.00467071	10000000	467.0715	= 100 mM

Notes on Singer and Bodell Nature Paper 1978

Digested 100ul = 1 mg DNA in Sterile Water

In Nature Paper:	ul	**USING 2/1/00 Working Stocks:** ul	
DNA @10mg/ml	100	300	(Buffer was used to prep DNA)
0.5 M Tris 7.3 buffer	20	0	
1 M MgCl2	2	0	
DNA Ase	20	80	Ours is 1/4 strength, use 4x
SVP	20	20	
Bact. Alk Phos	8	4	Ours is 2x units/ml, use 1/2
Acid Phosphatase	8	8	

DNAase I is 5 mg/ml, 2465 Units per mg, 12325 U/ml
SVP is 5 mg/ml, 32 Units per mg, 160U/ml
Alkaline Phos 5 mg/ml, 37 Units/mg, 185 U/ml
Acid Phosp 5 mg/ml, 16 U/mg, 80 units/ml
Worthington Biochemical Support recommended reconstituting enzymes with buffer and freezing at -20 or -80 C in small aliquots.
Could reconstitute with water, but may get unstable pH that lowers activity.

notes (cont.)

Working procedure for preparing master stocks according to Nat.	Enzyme (mg)	Tris Buffer* (ml)	mg/ml	U/ml
DNAase I 1400 U/mg Powder 25 mg	25	2.5	10	14000
SVP 20 units per mg dry wt, have 100 units=5mg	5	0.666	7.5	150
Alk Phos 39.9 units/mg protein, have 1 mg, 9.1 mg/ml, have 109ul, 360 u/	1	0.2	5	150
Acid Phosphatase 15 U/mg, have 1 g	5	1		75

Working procedure used for preparing Master Stocks (some made before paper was located)

	Label	**Enzyme (mg)**	**Tris Buffer* (ml)**	**mg/ml**	**U/ml**	**Buffer**
DNA	STDNA1	2.9	3.48	0.83		Not sterile, with $MgCl_2$
DNAase I 1400 U/mg Powder 25 mg	DP1	2.6	1.04	2.5	3500	Not sterile, with $MgCl_2$
SVP 20 units per mg dry wt, have 100 units=5mg	VPH1	5	0.737	6.78	150	Sterile, no $MgCl_2$
Alk Phos 39.9 u/mg prot, have 1 mg, 9.1 mg/ml, have 109ul, 360 u/ml	BAPF1	1	0.2	5	150	Sterile, no $MgCl_2$
Acid Phosphatase 15 U/mg, have 1 g	AP1	5	1	5	75	Sterile, no $MgCl_2$

Our other prep. Of Alkaline Phosphatase 35.9 u/mg P, 20.3 mgP/ml = 700 U/ml 5 mg got 0.246 ml

PREPARATION OF 0.1M TRIS BUFFER

Tris:
(Tris[hydroxymethyl]amino-methane)
$C_4H_{11}NO_3$

MW (g/mol) = 121.1

Weight = MW * Molarity

MW (g/mol)	Molarity (mol/L)	Cal Weight (g)	Act. Weight (g)
121.1	0.1	6.055	6.0503

*Only 500 mL of buffer was made, so the weight was adjusted.

To Prepare 1 liter 0.1M Tris Buffer

1 For a 1L solution weigh out 12.11 g of Tris
2 Place the weighed out Tris into a 1L bottle and add 1 L of sterile water*
3 Check the pH of the solution by using the pH meter to make sure it is at approx. 7.2
4 If the pH is too high add HCl until it is stabilized at 7.2
5 If the pH is too low add NaOH until it is stabilized at 7.2
6 Following the preparation of the buffer it is stored in the refrigerator.
*Water can be sterlized by putting it through a syringe filter or a more developed system.

Actual pH = 7.2

Notes: The buffer preparation of 2-3-00 was made with sterlie water. No $MgCl_2$ was used to prepare this buffer.
Also in future experiments we might like to use 0.1M buffer for the resuspension of the DNA and 0.5M buffer for the digestion reaction. A separate buffer containing 1M $MgCl_2$ will also be made for future experiments.
Add 95.21 mg to 100 ml of the 0.1M Tris Buffer

Original Set-up of stock solutions. **Only DNA and Dnase was set-up in this manner.**

HPLC INJECTION LOOP HOLDS 200ul. Will set up for 500 ul digestion mixes to allow for losses in filtration and replicate injections:

	DNA	DNA-ase	SVP	Alk P
Weight (ug)	250	250	100	100
Vol (ul)	300	100	50	50

		Stock		Vol of Stock Soln		Actual		Actual
	Conc.	ug	Buffer	ug	ml	ug		ml
DNA		**250**	**300**	**2500**	**3**	**2900**	833.3333	3.48
DNase		**250**	**100**	**2500**	**1**	**2600**	2500	1.04
SVP		100	50	1000	500		2	0
Alk P		100	50	1000	500		2	0

Notes: Original Set-up of stock solutions. **Only DNA and Dnase was set-up in this manner.** The following reagents were prepared in a separate manner described in Stock2.

PREPARATION OF STOCK REAGENTS FOR DIGESTION RXN

Date: 2-3-00

Specimen	Abbr.*	Label	Amt Enzyme (mg)	Tris Buffer (mL)	Conc (mg/mL)	U/mL	0.1M Tris Buffer** pH=7.2
Salmon Testes DNA	no	STDNA1	2.9	3.48	0.83		Not sterile, with $MgCl_2$
Deoxyribonuclease	DP	DP1	2.6	1.04	2.5	3500	Not sterile, with $MgCl_2$
Snake venom phosphodiesterase	VPH	VPH1	5	0.67	7.50	150	Sterile, with $MgCl_2$
Bacterial Alkaline phosphatase***	BAPF	BAPF1	1	0.2	5	150	Sterile, with $MgCl_2$
Wheat germ (acid) phospatase	AP	AP1	5	1	5	75	Sterile, with $MgCl_2$

*Abbreviations are from Worthington Biochemicals labeling system.

**Tris Buffer used for DNA and DNase was 0.1 M Tris and 0.01 $MgCl_2$. The remaing three enzymes were prepared in 0.1M Tris Buffer with sterlie water and without $MgCl_2$.

***BAPF was not put into buffer, bc it is already in solution and its concentration was correct. The amount needed was taken directly from the original vial.

Notes: Each reagent (except BAPF) was pipetted into individual aliquots and stored in the -80 freezer. This was done to prevent the loss of activity by the thawing/refreezing process and also recommended by the technical support of Worthington.
A box (it is labeled DNA ADDUCT ASSAY DNA DIGESTION) containing all the reagents (except BAPF1) is in the -80 freezer, second shelf from the bottom. BAPF is stored in refrigerator A. There is enough enzymes to perform 8 separate digestions. There is an excess of both VPH1 and AP1 and these enzymes are stored in the -80 freezer.

DNA DIGESTION MIX

2/3/00

Components	Volume (uL)
STDNA1	300
0.1M Tris Buffer w $MgCl_2$*	
DP1	80
VPH1	20
BABF1	4
AP1**	8

*already mixed with enzymes
**not mixed with buffer

Components	Volume (uL)
DNA digested 2-1-00	100
0.1M Tris Buffer w $MgCl_2$*	
DP1	80
VPH1	20
BABF1	4
AP1**	8

*already mixed with enzymes
**not mixed with buffer

Digestion Mix for Future Experiments:

# of Samples = 8

Components	Volume (uL)
Specimen	
0.5M Tris Buffer	800
1M $MgCl_2$	16
DP1	640
VPH1	160
BABF1	32
AP1	64
total	1712

Following the set up the tubes were put in the incubator/shaker at 37 degrees for 24 hours.

In the future will mix DP1 up 4x as concentrated - use 20 ul

0.1 M Tris Buffer, containing $MgCl_2$ was used for this digestion.

INACTIVATION & PURIFICATION

Following the digestion the mixture is:
1. Heated at 100 degrees for 90s
2. Passed through a centrifugal filter with a pore size of 0.2 um

FILTRATION

Filter Hydrolysate with 0.2 um centrifugal microfilters at 1300 g for 5 min

SOP-2

SOP FOR THE COMET ASSAY

CONTENTS:

I. PREPARATION OF REAGENTS

a) Hanks Balance Salt Solution with 20 mM EDTA
- To 400 ml HBSS (Ca^{+2}, Mg^{+2} free), add 3.72 g EDTA set pH to 7.5, q.s. to 500 ml.
- Store at 4 degrees Celsius.
- Use: tissue isolation

b) Phosphate Buffered Saline (Ca^{+2}, MG^{+2} free)
- Dolbecco's PBS - 1L packet, add 3.72 g EDTA set pH to 7.4, q.s. to 1000 ml, filter sterilize.
- Store at 4 degrees Celsius.
- Use:

c) Lysing Solution
- Ingredients per 1000 ml:

MAKE THE DAY BEFORE:

2.5 M NaCl -	146.1 gm
100 mM EDTA -	37.2 gm
10 mM Tris -	1.2 gm
solid NaOH -	6.5-7 gm (to set pH to 10)
1% Na Sarcosinate -	10.0 gm

q.s to 890 ml with dH_2O, store at room temperature

DAY OF EXPERIMENT:

1% Triton X-100	10.0 ml
10% DMSO	100.0 ml

NB-remember to refrigerate prior to slide addition for 30-60 min!

d) Electrophoresis Buffer
- 300 mM NaOH / ! mM EDTA
- Prepare from stock solution:

10 N NaOH - 40 g NaOH to 100 ml CEPB treated water (good for ~2wk).

-For buffer; add 30 ml 10 N NaOH and 2 ml of 0.5 M EDTA to a final volume of 1000 ml in CEPB treated water (make fresh just before use).

e) Neutralization Buffer
- 0.4 M Tris, pH 7.5
- 63.04 g Tris (acid), q.s to 1000 ml with dH_2O
- Set pH using NaOH
- Store in refrigerator

f) Staining Solution

- Ethidium Bromide (10x Stock - 20 ug/ml)
- 10 mg in 50 ml dH_2O
- Store at room temperature
- For 1 X stock - mix 1 ml with 9 ml dH_2O

II. CELL ISOLATION

a) Whole Blood

- Mix 5 ul whole blood with 75 ul Low Melting Point Agarose, layer onto slide.

b) Isolated Lymphocytes

- Mix 20 ul whole blood with 1 ml RPMI 1640 in a micro centrifuge tube, add 100 ul Ficoll below the blood/ media mixture. Spin for 3 min at 2000 xg. Remove 100ul of middle top layer, add to 1 ml media and mix, spin for 3 min to pellet lymphocytes. Pour off supernatant, resuspend pellet in 75 ul Low Melting Point Agarose, layer onto slide.

c) Frozen Lymphocytes

- Thaw frozen Lymphocytes
- Put cells into a 15 ml tube. Add media (type varies) without serum to 15 ml.
- Centrifuge at 100xg for 10 min at 4 degrees Celsius
  Using Beckman GH-3.8 rotor with buckets: 650 RPM = 96.5 xg
- Repeat wash with serum free media
- Resuspend in 2 ml Phosphate Buffer Saline
- Hold on ice, prepare for treatment if any.
- Count cells and determine cellular viability.

d) Tissue Isolation (mice)

(liver, spleen, lung, bone marrow, brain, testis, etc.)

- Remove a small piece of the organ, place in 1 ml of cold HBSS with 20 mM EDTA on ice. Mince into fine pieces, let settle, remove 5 ul cell suspension, add to 75 ul Low Melting Point Agarose, layer onto slide, For easily desegregated tissues (e.g. spleen and testes) add two to three ml more HBSS and remove 5 ul for slides. For liver only, mince into large pieces, let settle, aspirate HBSS, add 1 ml fresh HBSS, mince into finer pieces. Add 5-10 ul cell suspension to 75 ul LMPA, layer onto slide. For bone marrow, perfuse femur with one ml cold HBSS into a microtube, remove 5 ul cell suspension for slides.

e) Culture Isolation

- 1. Monolayer cultures - scrape off cells into the culture media in the cell dish using a teflon scrapper (Do not trypsinize cells) to yield approximately $1x10^6$ cells/ml. Add 5 ul cell suspension to 75 ul LMP Agarose, layer onto slide.
- 2. Suspension Culture - Add 10 000 cells in 10 ul or less volume to 75 ul LMP Agarose, layer onto slide.

III. CELL COUNT USING A HEMOCYTOMETER

After isolation of cells it is important to estimate the concentration of cells you have. The amount of cells needed for each slide is ca 10 000. i.e. in order to know whether you have enough cells for the assay or not, take the number of slides to be made and multiply with 10 000. This value of total number of cells will also give you an estimate of how to dilute your assay sample with cells.

Ex:

Lymphocytes are treated with 3 different dilutions of the H_2O_2. From each dilution we will layer 4 slides. After counting the # of lymphocytes, we estimated the volume needed from the concentrated solution of cells in order to have 40000 cell/ml in each tube.

(1) Count cells and determine cellular viability by mixing in a separate Eppendorf tube:

100 ul Trypan Blue solution
60 ul HBSS
40 ul cell suspension

(2) Count cells of 5 squares of both grid sections.
(3) Count the # of Live (unstained) and Dead (dead) cells.
(4) Then calculate your concentration of cells by taking the total # of cells for all the ten squares and divide by 10. Multiply by the dilution factor of 5. This value times 10^4 is the # of cells per ml of the cell suspension;

Cells/ml = average count of viable cells per square x 5 (dilution factor) x 10^4

Cells/ml = _____ x 5 x 10^4 = _____

Cell viability = total viable cells/total cells x 100

(6) After cell counts are determined, decide the volume of the cell suspension is needed to put the necessary number of cells into the assay system.

IV. PREPARATION OF TREATMENT CONDITION

Each test tube must have a final total volume of 1 ml. After adding the volume of cells and the 10 ul of treatment solution, aliquot Phosphate Buffer Saline or media to 1 ml. Work in dark to protect cells and drug from light. Avoid creating bubbles.

a) Determining Concentration of Treatment

(1)The concentration of the drug or the solvent treatment to be used should not be more than 1 % of your assay system. That is 10 ul in a 1 ml total volume. Since 1 % is one part in one hundred, your stock treatment solution must be 100 times more concentrated than your desired concentration.

- Ex. 1:

If 300 uM BCNU is the desired concentration for the assay, multiply by 100 to get the value 30 000 uM which is 0.03 M. Next calculate the weight (gr) of drug in solvent needed in order to obtain the 100x conc., using the formulas: $C_1V_1 = C_2V_2$, C = mole/V, Mole = molecular weight/gram.

- Ex. 2:

Using hydrogen peroxide (H_2O_2), determine the stock concentration being used. H_2O_2 mw: 34.01 g/mole.
Stock-Sigma: 30.6% = 30,6g/100 ml
3.6 g/0.1L x 1 mole/34.01 g = 8.997M
Then calculate the amount needed to obtain your 100x desirable concentration. Typically the volume of H_2O_2 is very small. For more accurate concentration using the final solution volume being 10 ml, take out the calculated valve H_2O_2 in solvent from the prepared tube holding 10 ml solvent and next add the same amount H_2O_2.

(2) When the solvent and the drug is mixed, action must be quick. Have ready the assay tubes with the cells suspended in growth media ready for reception of drug. After drug is added to each tube, the tubes should be quickly inverted in order not to create a gradient where the cells at the surface are exposed to drug for longer time than cells throughout the assay tube.

(3) Incubate the tubes with treatment at desired conditions of time and temperature.

(4) After incubation, centrifuge at 100 xG for 10 min at 4 degrees Celsius. Using Beckman GH-3.8 rotor with buckets: 650 RPM = 96.5 xG.

(5) Decant the supernatant and resuspend in 0.5% Low Melting Point Agarose. A

volume of 75 ul of LMPA is needed for each slide to be made out of the assay tube.

(6) Layer slides (see section "Preparation of Slides for the SCG assay")

b) Endonuclease

-Treat slides with endonuclease III for 1hr at 37 degrees Celsius in the incubator. Place slides in glass trays that have 1 ml pipettes along the bottom to hold the slides above the surface. Beneath the pipettes a clean kimwipes to hold DEPC water to help humidify the tray. After the slides are carefully placed on the supportive pipettes, use a Pasteur pipette to drop the endonuclease solution on the slides, approx. 1ml per slide. Cover the tray with plastic wrap to keep from drying out.

c) Repair

- For some assays it is interesting to look at the capability of cells to repair after treatment as opposed to cells analyzed right away.. Hence, we allow one set of treated cells layered on slides to sit for 30-60 min at 36 degrees Celsius in a water incubator to allow for any repair

V. PREPARATION OF SLIDES FOR THE SINGLE CELL GEL ASSAY

Prepare 0.5 % Low Melting Point Agarose and Regular Agarose in Calcium and Magnesium free PBS (125 mg in 25 ml). Microwave or heat until near boiling and the agarose dissolves. Aliquot 5 ml samples into scintillation vials and store at 4 degrees Celsius until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37 degrees Celsius water bath to cool.

Already prepared slides with one layer of normal melting point agarose in PBS are used for the layering of the cell suspension .

(1) Make 1% normal melting point agarose in PBS.
(2) While agarose is hot, dip conventional slide in a coplinjar up to one half the frosted area and gently remove.
(3) Wipe backside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed for quicker drying.
(5) Store the slides at room temperature until needed. Avoid high humidity conditions, if possible.

After the assay tubes with cells have undergone incubation with treatment.

(1) Centrifuge at 100 xG for 10 min at 4 degrees Celsius.
Using Beckman GH-3.8 rotor with buckets: 650 RPM = 96.5 xG.

(2) Decant the supernatant and resuspend in 0.5% LMPA. A volume of 75uL (containing 10 000 cells)ul is needed for each slide.

(3) Keep in water bath at 37 degrees Celsius.

(4) Layer slides

Layering of the slides:

(1) Work in dim light to prevent DNA damage.

(2) On to a slide previously coated with agarose and dried, place a cover slip at a 45 degree angle holding it while adding carefully 75 ul of the agarose and cell suspension to the inside surface of the cover slip. Carefully use the pipette tip to easily lay the slip down onto the slide (no bubbles).

(3) Place slide on ice (minimum of three minutes).

(4) Gently slide off the coverslip and add a third agarose layer consisting of 75 ul LMPA to the slide. Replace coverslip and return to ice tray.

VI. LYSIS

- Remove coverslip and slowly lower slide into cold, freshly made Lysing Solution. Protect from light and place in 4 degrees Celsius refrigeration for a minimum of 3 hours. Slides may be stored for extended periods of time in cold Lysing Solution (at least 4 weeks) without affecting migration.

VII. ELECTROPHORESIS AND NEUTRALIZATION

(1) Make sure the electrophoresis tank is clean, rinse in distilled water.

(2) Gently remove the slides from the Lysing Solution. Place slides on the horizontal gel box near the anode (+) end, sliding them as close together as possible. Always work with 24 slides at the time, if less you have to fill in the empty space in the electrophoresis tank with blank slides.

(5) Fill the buffer reservoirs with freshly made Electrophoresis Buffer until liquid level completely covers the slides (avoid bubbles over the slides).

(6) Let slides sit in the alkaline buffer for 20 minutes to allow unwinding of the DNA and the expression of different classes of alkali-labile damage.

(7) Turn on power supply to 25 volts and adjust current to 300 mAmp. by slowly raising or lowering the buffer level. Depending on the purpose of the study and the extent of migration in control samples, allow slides to run for 10 to 40 minutes.

(8) Turn off the power. Gently lift the slides from the buffer and place on a staining tray. Dropwise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Repeat two more times.
(9) Drain slides, add 50 ul 1x Ethidium Bromide stain and cover with a fresh coverslip.
(10) Before viewing the slide, blot away excess liquid on the back and edges.
Note: During the electrophoresis process work under dimmed lights.

IX. EVALUATION OF DNA DAMAGE

(1) For visualization of DNA damage, observations are made of ethidium bromide stained DNA at 40x magnification using a fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm.
(2) We use Confocal Comet or Leca Quantiment 520 image analysis system linked to CCD camera to quantitate DNA image length, DNA image diameter, nuclear diameter, tail intensity, and tail moment. Generally, 50 randomly selected cells per sample are analyzed.

SOP 3 Trevigen CometAssayTM Reagent Kit for Single Cell Cel Electrophoreisis

From the Trevigen CometSlideTM Manual

Materials Supplied with Kit
2 x 500 ml Lysis Solution
15 ml Comet LMAgarose (LMA)
25 Trevigen CometSlidesTM
12.5 ml 200 mM EDTA, pH 10
5 ul SYBR Green Concentrate

Material/Equipment Required But Not Supplied
Same equipment as the Original Comet Assay

Reagents:
10X PBS, Ca++ and Mg++ free
NaOH Pellets
0.5 M EDTA (ph 8.0)
Ethanol
TE Buffer (10mM Tris (pH 7.5), 1 mM EDTA
Deionized water

Reagent Preparation
Reagents marked with an asterisk (*) should be prepared immediately before use.
Wear gloves, lab coat, and eye protection when handling any chemical reagents.

1. 1X PBS, Ca++ and Mg++ free
Dilute 10X PBS with deionized water to prepare 1X PBS. Store at room temperature.

2. Lysis Solution
For up to 10 slides, use 40 ml of the prepared lysis solution and add 4 ml of DMSO for samples containing heme, such as blood cells or tissue samples. Lysis solution contians 2.5 M Sodium Chloride, 100 mM EDTA pH 10, 10 mM Tris Base, 1% Sodium Lauryl Sarcosinate, and 1% Triton X-100.

3. Comet LMAgarose
The Comet LMAgarose is ready to use when molten. Loosen cap to allow for expansion then heat bottle in a 90-100 C water bath for 5 minutes and then place into a 37 C water bath for at least 20 minutes to cool. The agarose will remain molten for smaple preparation indefinitely.

4. Alkaline Solution, pH>13*

Wear cloves when preparing and handling this solution.
Per 50 ml of alkaline solution combine .6 g NaOH pellets, 250 ul of 200 mM EDTA, and 49.75 ml of dH20
Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use.

5. Alkaline Electrophoreisis Solution pH>13 (300 mM NaOH, 1 mM EDTA)

Prepare a stock solution of 500 mM EDTA, pH 8.
For 1 liter of electrophoreisis solution add 12 g of NaOH pellets and 2 ml of 500 mM EDTA, pH 8 to 1 liter of dH20
Adjust the volume prepared based on the dimensions of your electrophoresis apparatus. Use of freshly made solution is recommended.

6. SYBR Green Staining Solution

Add 1 ul of SYBR Green Concentrate to 5 ml of TE buffer.
The diluted stock is stabe for several weeks whe stored at 4 C in the dark.

Final protocol for using the slides from Trevigen

Preparation of MCF-7 Cells:

1. To prepare the MCF-7 cells first warm up media to 37 C in 3/4 full water bath: EF, 5% FBS/EF, FBS, and 0.05% Trypsin-EDTA. Then take out a T25 flask with the growing cells in it out of the incubator and pour away old medium from the flask in to beaker with bleach (waste). Rinse with 1 ml of Trypsin and pour into waste beaker, then repeat. Add 1 ml Trypsin to flask an incubate at 37 C for 7-10 minutes. Add 4 ml EF and wash bottom of flask. Pipette up and down until you get an even cell suspension. Transfer suspension to a 15 ml centrifuge tube containing 1 ml FBS and centrifuge for 310xg for 5 minutes. Remove the supernatant and resuspend the cells in 1 ml EF. Count the cells.

2 Cell count is determined by Trypan blue exclusion by the following:

90 ul Trypan blue

10 ul cell suspension

Mix by pipetting in a well and let stand for 5 min. and count on a hemacytometer

Count the four side squares and take the average of all of these squares. Then multiply the average x 10 x 10e4.

3. Now determine the volume of cell suspension needed to put the necessary number of cells into the assay system or you can just alliquot the sample into the separate tubes. Add the volujme of cell suspension to start the experiment with 100,000 cells/tube.

4. Bring up the volume of each assay tube. Noting volume of cell suspension in each assay tube and that 1% of the final volume is the drug in it's solvent: The rest of the volume should be filled with growth medium to equal 1 ml.

Procedure for Determining Drug Treatment Dosage Using Mettler H54AR:

Planned drug exposures: 0uM 10uM 30uM 100uM

The Drug Calculations:

1. Weigh the proper amount of drug needed:
   a. Weigh vials
   b. Adjust coarse adjustment weight using "1/2"
   c. Fine tune on "1" (bars between teeth)
   d. Remove static (when necessary) using static gun
   e. Take vial to chemical exhaust hood
   f. Using a spatula take and add small amount to respective tube
   g. Close tube and weigh on Mettler; weigh on "1"
   h. Get weight in milligrams and convert into micrograms. To find the amount of DMSO solvent that needs to be added to make a 1 M standard use the formula: Weight in g/Molecular Weight of BCNU = ml solvent (DMSO) needed for 1M

Set up the dilutions and label all of the tubes according to the BCNU Rx chart that is attached.

2. To do serial dilutions, first label all vials:B1M, B100, B100, B10, B10, B1, and DMSO only (control)

Pipette Required Solvent into each vial: 270, 350, 270, 350, 270, and 350

Use B1M to make first dilution 30ul

Use B100 to make next two dilutions using 150ul and 30ul of stock solution
Use B10 to make next two dilutions using 150ul and 30ul of stock solution
Use B1 for last Dilution with 150ul of stock solution
This will give you the new mM concentration now you can relabel your vials to correspond with these new concentrations. (B100, B30, B10, B3, B1, B0.3, and DMSO only (control)) Once the drug treatment stocks are made up they can be stored in the freezer for use in later experiments. When the solvent is added to the drug and mixed, action from there must be quick.

Adding the drug treatment stocks to the MCF-7 Cells:

3. Have ready the assay tubes with cells and RPMI 1640 growth medium ready for the reception of the drug. Now you add the drug treatment stock solutions to the corresponding tubes so that the drug is never 1% more than the total volume in the tube because the final concentration of the dilution is 100 fold of these drug treatment stocks. Considering this, the treatment stock solution must be 100 times more than the desired concentration (multiply by 100 to the molar concentration) Example, when 10ul is added to the assay tube the final concentration of the treatment drug in the total of 1000 ul will be the desired level.

4. After the drug is added to each assay tube, the tubes should be quickly inverted in order not to create a gradient where the cells at the surface are exposed to drug for longer than the rest of the cells throughout. After all tubes have been inverted invert tubes again. Work in the dark in order to protect the cells from light. Always be careful never to foam the suspensions. Place tubes in holder and Incubate assay tubes at 37 degrees C for two hours in an incubator. After treatment the tubes should be spun down at 210xg for 10 minutes at 4 degrees C.

Immunocytochemistry with Antibodies:

Note: Turn on the slide warmer and put the slides and pipette tips on it to warm up before adding the samples to the slides.

1. Resuspend the bead pellet in 100 ul (PBS+0.1%BSA+**Cd**+0.1%Saponin) and add 10 ul Superblock and incubate on ice for 5 minutes. Spin down the tubes at 210xg for 10 minutes and then remove the supernatant.
2. Resuspend in 100 ul (PBS+BSA+Cd+0.3%Saponin).
3. Add 10 ul of the Glutathione S-Transferase pi, mu, or alpha antibodies to the tubes and incubate 1 hour on ice.
4. Wash cells with 100 ul (PBS+BSA+Cd+0.1%Saponin). Spin down the tubes at 210xg for 10 minutes and then remove the supernatant and pick up in 100 ul (PBS+BSA+Cd+0.3%Saponin).
5. Add 2 ul secondary antibody (488 GAR secondary) and incubate 1 hour on ice.
6. Wash cells with 100 ul (PBS+BSA+Cd+0.1%Saponin) . Spin down the tubes at 210xg for 10 minutes and then remove the supernatant and pick up in 1 ml in ice cold 1X PBS (Ca++ and Mg++ free).

The Comet Assay:

Preparation for the Comet Assay:

Prepare all solutions according to the Trevigen manual before you start, use fresh solutions each time.

1. Prepare Lysis solution and chill at 4 degrees C or on ice for at least 20 minuntes before use.

2. Melt LMAgarose in a beaker of boiling water for 5 minutes with the cap loosened. Place bottle in a 37 degree C water bath for at least 20 minutes to cool. The temperature of the agarose is critical or the cells may undergo heat shock. Heat bloscks are not recommended for regulating the temperature of the agarose.

3. Combine cells at 1*10^5/ml with molten LMAgarose at a ratio of 1:10 and immediately pipette 75 ul onto CometSlide. If necessary use side of pipette to spread agrose/cells over the sample area. When working with many samples it may be convenient to place alliquots of the molten agarose into prewarmed microcentrifuge tubes and place the tubes at 37 degree C. Add cells to one tube, mix by gently pipetting once or twice, then transfer 75 ul alliquots onto each sample area as required. Then proceed with the next sample of cells.

Comet LMAgarose	500 ul
Cells in 1X PBS at 1*10^5/ml	50 ul

Note: If sample if not spreading evenly on the slide, warm the slide at 37 degre C before application>

4. Place slides flat at 4 degrees C in the dark (coldroom) for 10 minutes. A 0.5 mm clear ring appears at edge of the sample area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity enviornments. Locate the cells stained with GST with flourescent microscope/LSC using the Argon (green) laser.

5. Immerse slides in prechilled Lysis solution and leave on ice, or at 4 degree C (coldroom) for 30-1 hour or leave overnight.

6. Tap off excess buffer from the slide and immerse in freshly prepared Alkaline solution, pH>13. Wear goves when preparing or handling this solution. Leave CometSlidein Alkaline Slution for 20-60 minutes at room temperature, in the dark.

Alkaline Electrophoresis:

7. Tranfer slide from Alkaline solution to a horizontal ectrophoresis apparatus. Attach the hosing so that the cooling unit is connected to the electrophoresis unit. Set the cooling unit to less than 10 degrees C. Set the slides onto the platform of the electrophoresis chamber. Set them all in the same direction and allign equidistant from the electrodes. Carefully pour the Alkaline Solution until the level just covers samples. Set the voltage to about 1 Volt/cm. Add or remove buffer until the current is approximately 300 mA and perform electrophresis for 20-40 minutes.

8. Gently tap off excess electrophoresis solution, rinse by dipping several times in dH20, then immerse in 70% ethanol for 5 minutes.

9. Air dry samples. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room teperature, with desiccant prior to scoring a this stage.

10. Place 50-100 ul of diluted SBR Green or To-Pro 3 onto each circle of dried agarose and icubate at 37 degree C for 10 minutes.

11. Rinse slides 3 times by submerging them in 50 ml H20.

12. Cover with 50 ul antifade (Prolong) and coverslip. For long storage seal sides of the coverslip with clear nailpolish. Slides may be stored in the dark for a few days. Scan the slides on the LSC to find the cells and score the comets.

SOP 4 Suggested Formulation for EF media (2X)

(Prepare all media with the most purified water possible)

E-Medium = 1 L (EMED)

1 Packet Dulbecco's Modified Eagles's Medium, 1000 mg/l glucose (Gibco No. 074-1600)
2.38 g HEPES
2.86 $NaHCO_3$
10 ml Amino Acid Vitamin Supplement, contents listed below (added after most of the H_20 has been added) (A.A)
6.5 µl α-thioglycerol (Sigma)
1 ml Trace Elements B, contents listed below (Mediatech)
1 ml Penicillin-Streptomycin lypholized antibiotics (Gibco)
80 µl Na_2SeO_3 ($1.25x10^{-3}$ M stock solution) Make to 1 l – check pH (6.9-7.0), Filter and store at 4 C in dark

F-Medium – 1 L (FMED)

1 packet Ham's nutrient mixture f-12 (Gibco No. 074-1700)

2.38 g HEPES

0.336 g $NaHCO_3$

6.5 µl α- thioglycerol (Sigma)

1 ml Trace Elements B (same as above)

1 ml Antibiotics (same as above)

80 µl Na_2SeO_3 (same as above)

Amino Acid – vitamin supplement for EMED (100X)

1.	L-alanine	250 mg/100 ml
2.	L-asparagine.H20	500 mg/100 ml
3.	L-aspartic acid	300 mg/100 ml
4.	L-cysteine	700 mg/100 ml
5.	L-glutamic acid	750 mg/100 ml
6.	L-proline	400 mg/100 ml
7.	Sodium pyruvate	1100 mg/100 ml
8.	Vitamin B-12	0.25 mg/100 ml
9.	Biotin	0.3 mg/100 ml

Recipe for Amino Acid Supplement:

Dissolve each in 5 ml purified water. Numbers 3 and 5 require addition of 5 N NaOH; no. 4, several drops of concentrated HCl; no. 2 and 3, warming to dissolve (it takes time and patience to them all into solution). Mix together, adjust pH to 2.0 and bring volume up to 100 ml with water. Filter and store at –20 C in 10 ml aliquots.

Trace Elements (TE) 1,000,000X stock

	Concentrations	Weigh/100 ml water
1. Na_2SiO_3* 9 H_20 (FW=284.2)	$5x10^{-1}$ M	14.2 g
2. $MnSO_4$* 1 H_20 (FW=169)	1x10-3 M	16.9 mg
3. $(NH_4)_6Mo_7O_{24}$ * 4 H_20 (FW=1236)	1x10-3 M	124 mg
4. NH_4VO_3 (FW=117)	5x10-3 M	58.5 mg
5. $NiCl_2$* 6 H_20 (FW=237.7)	5x10-4 M	11.9 mg
6. $SnCl_2$*2 H_20 (FW=225.6)	5x10-4 M	11.3 mg

(all from Merck or Fluka)

TE stock is made by taking 10 µl of of 1-6 above and adding to 0.940 ml of EF.

The EF media is a 1:1 mixture of EMED and EFED mentioned above.

Either mix up separately or add up the amounts of the same ingredients for each media type and make the media up all together at one time. Follow the recipe below to make the media. Makes 1 L of concentrated growth media, to be used at half strength by diluting with an equal part of sterile H20 for normal use, but at full strength (2X) in methyl cellulose mixture.

Recipe for making up the EF media:

Dissolve dry ingredients in about 0.8 L water in volumetric flask. Add remainig 4 liquid ingredients. Bring volume up to 1.0 L with sterile H_20. Adjust pH to 6.9. Filter solution through bottle top filter with 0.02 µ filter into 500 ml bottles and cover with foil to protect from light.

Storage: 4 C

SOP #5 *Maintaining MCF-7 WT and AdrR cells*

Use either T25 or T75 flasks. Split one flask into two, keep one flask until the next passage as reserve. If subculture is made successfully, no contamination, you may discard the reserved flask. **Keep sterile conditions**. Use clean pipettes; don't touch tips. Work with clean hands, do not move your hand above opened bottles or flasks. Always keep open bottles and flasks in middle of hood. Take care to only open flasks, pipettes, and tubes directly in hood.

1) Warm up media to 37 C in ¾ full water bath: EF, 5% FBS/EF, FBS, and 0.05% Trypsin-EDTA.
2) Pour away old media from flask into beaker with bleach (waste).
3) Rinse with 1 mL of Trypsin and pour into waste beaker. Repeat.
4) Add 1 mL of Trypsin to T25 flask, 3 mL of Trypsin to T75 flask and incubate at 37 C for 7-10 minutes.
5) Add 2 mL EF (no FBS) and wash bottom of flask. Pipette up and down until you get an even cell suspension.
6) Transfer cell suspension to a centrifuge tube (15 mL tube) containing 1 mL FBS for T25 or 2 mL FBS for T75.
7) Centrifuge cells at 2,000 RPM for 5 min.
8) Pour away supernatant and flick cells with your finger. Add 2 mL EF to pellet and mix up cell suspension with pipetting.
9) Add 5 mL FBS/EF media to T25 flask or 15 mL FBS/EF media to T75 flask.
10) Add 1 mL of cell suspension to a newly labeled flask (date, cell type, and passage number).
11) Place in incubator, MAKE SURE DOOR IS SHUT TIGHTLY!

<u>To start a new line:</u>

Remove cryogenic vial from liquid Nitrogen. Place vial in water bath and allow to thaw quickly. As soon as it is thawed, pour into 15-mL tube. Slowly dilute with cold 5% FBS/EF. Add media dropwise to the thawed solution. Spin down at 2000 RPM for 5 minutes. Continue from step 8.

SOP 6. Spiking Experiment Separating MDA Breast Cancer Cells from Bone Marrow Samples Using the OncoQuick Method

Equipment

Graduated Cylinder
.22 μm bottle top filter
Swing bucket rotor centrifuge
Cytospin centrifuge
Hemacytometer
Electron Microscope

Materials

Solutions

1 x sterile washing buffer working solution
Preparation of .5L of 1 x sterile washing buffer working solution: 400 ml of sterile distilled H_20 mixed with the contents of the 5 x washing buffer concentration, bring the volume up to .5 L with distilled H_20 and pass the washing buffer through a 0.22 μm bottle top filter, store solution at 4-22°C.
Trypan Blue
TBS Buffer

Samples and Cell Lines

Bone marrow sample from patient with breast cancer with little or no tumor cells
Cells from the MDA breast cancer cell line

Pipettes

1 and 5 ml Serological pipettes
1 ml pipette

Tubes

OncoQuick 50 ml tubes
5 ml centrifugation tube
15 ml centrifugation tube

Other materials

Slides
Cover slips
Attachments for the cytospin slide
Well plate
Latex gloves
Biohazard waste bags
Labels to mark tubes and washing solution

Procedure for Spiking Experiment

1. Make up the washing buffer working solution according to the above directions.

2. Pre-cool the centrifuge to 4°C and prepare a bucket filled with ice.

3. Incubate OncoQuick tube and bone marrow samples for 10-15 minutes on ice.

4. Add 90 µl of Trypan Blue and 10 µl of the bone marrow sample to a well plate and mix with the pipette. Insert the mixture into a hemacytometer and count the number of bone marrow cells on the 4 outside squares of the grid. Then take the average of the four counts and multiply by 10 x 10^4 to get the number of cells/ml of the sample.

5. Repeat this procedure with the MDA cells and then decide on the ratio of MDA cells to add to the sample. Use about 10,000 MDA cells with 1-ml bone marrow sample. To find out how many mls of cells are needed to get 10,000 cells, divide 10,000 by the number of cells/ml found from the hemacytometer. Mix the bone marrow and MDA cells together in a 5-ml tube.

6. Pipette out 1 ml of the separation medium through the porous barrier into a 5-ml centrifugation tube. Carefully over-layer the MDA cell spiked bone marrow sample into the tube with the separation medium. Slightly incline the tube and introduce the sample slowly down the side of the tube.

7. Spin sample-filled 5 ml centrifugation tube at 1600 x g (2800 RPM) at 4°C for 20 min in a swing bucket rotor with slow acceleration and no brake.

8. After centrifugation, the tumor cells will be in the interphase between the upper plasma (yellowish/brownish) and the lower separation medium (blue). Usually this cell fraction is not visible. Gently collect cell layer by using a serological pipette and transfer to a fresh 15-ml conical/pointed bottom centrifugation tube with cap.

9. Add 1 x washing buffer to bring the volume to 5-ml. Cap tube and mix cells by gently inverting the tube 5 times.

10. Pellet the cells at 200 x g for 10 min. Gently aspirate as much supernatant as necessary without disturbing the cell pellet. Resuspend cell pellet in 1 ml of TBS buffer for the subsequent procedure.

11. Make a cytospin slide and spin in a cytospin centrifuge. Then stain the separated cells with cytokeratin to see how many tumor cells were recovered.

SOP 7 DynalBead Separation

From Dynal manual

Protocol 1: For Keeping Beads Attached to Tumor Cells

Protocol 2: Removal of Beads from Tumor Cells

PRODUCT DESCRIPTION

CELLectionTM Epithelial Enrich is based on the CELLectionTM Positive Isolation System with Universal Detachment. The CELLectionTM Dynabeads® are uniform, superparamagnetic, polystyrene beads (4.5 µm diameter) coated with antibody via a DNA linker to provide a cleavable site for cell detachment. The antibody coated onto the CELLectionTM Dynabeads is an anti-EpCAM (epithelial cell adhesion molecule) mouse IgG1 monoclonal (Ber-EP4) specific for two (34 and 39 kDa) glycopolypeptide membrane antigens expressed on most normal and neoplastic human epithelial tissues (1). The reactivity of this antibody is identical to mAb HEA125. The product is especially designed for the enrichment of circulating tumour epithelial cells from human peripheral blood and bone marrow. It may be used for isolation of epithelial cells from tissue digests and other compartments.

The CELLectionTM Dynabeads are supplied as a suspension containing 4 x 108 beads/ml in phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN3).

PRINCIPLE

Immunomagnetic cell isolation using CELLectionTM Epithelial Enrich provides a simple and reliable method for positive isolation of circulating tumour epithelial cells. The cells can be enriched directly from whole blood, bone marrow or from mononuclear cells (MNC) as obtained by density gradient centrifugation of peripheral blood or bone marrow. During a short incubation period, the epithelial cells bind to CELLectionTM Dynabeads, and subsequently, the rosetted cells can be isolated and washed using a magnet (e.g. a Dynal Magnetic Particle Concentrator (Dynal MPC®)). After cell isolation, the DNase- containing Releasing Buffer is added to detach the beads from the cells. Circulating tumour epithelial cells are extremely rare and may be only present at a frequency of 1-10 in 106 MNC. In model experiments, CELLectionTM Dynabeads consistently give excellent recovery of the seeded cells. The cells were enriched up to 5 logs with 70% yield of tumour cells. The enriched cells are viable and may be cultured directly and used for immunocytochemical staining with EPiMET® Enrich.

Buffers/solutions

Phosphate buffered saline (PBS) pH 7.4:
NaH2PO4 x H2O 0.16 g
Na2HPO4 x 2 H2O 0.98 g
NaCl 8.10 g
Distilled water to 1 litre

Washing buffers:

PBS with 0.1% (w/v) bovine serum albumin (BSA) / 0.6% Na citrate
PBS with 0.1% (w/v) bovine serum albumin
PBS with 20% (w/v) fetal calf serum (FCS)
RPMI 1640 with 1% fetal calf serum (FCS)

All reagents should be analytical grade.

The fetal calf serum should be heat inactivated for 30 minutes at 56°C and tested for cytotoxicity before use.

Use FCS in buffers for cell enrichment steps from MNC samples only. Use BSA (0.1%) in buffers for cell enrichment steps from blood or bone marrow. Do not use FCS with blood or bone marrow as it can cause clotting. For the DNase releasing step the buffer used is RPMI/1% FCS.

ADDITIONAL MATERIALS NEEDED

Materials that are not included, but are needed to perform cell isolation procedures with CELLectionTM Epithelial Enrich:

Magnetic separation device (For all work with Dynabeads, Dynal Biotech recommends the use of a Dynal magnet, Dynal MPC). The choice of Dynal MPC is dependent on the number of samples and the volumes tested at one time. Also, protocols involving mRNA isolation (in addition to cell isolation) require magnet units (Dynal MPC) that accommodate microcentrifuge tubes.

Mixing device that allows both tilting and rotation of test tubes (e.g. Dynal MX 1, 2 or 3, Dynal Sample mixer
Test tubes for cell isolations
Pipettes
Washing solutions (PBS / 0.1% BSA / 0.6% Na citrate and PBS / 0.1% BSA and RPMI / 1% FCS and PBS / 20% FCS).

For materials required to perform the immunocytochemistry with EPiMET® Enrich, please refer to the package insert provided with EPiMET® Enrich (Prod. No. 160.01).
For protocols and materials required for running control samples, see section on Controls below.

INSTRUCTIONS FOR USE

Reconstitution of Releasing Buffer
For each vial of freeze-dried DNase, transfer 300 µl buffer from the CELLectionTM Releasing Buffer (component 2) to each tube of DNase (component 1). Dissolve the enzyme gently.
Aliquot the reconstituted Releasing Buffer into suitable portions. Store at -20°C. Thaw immediately before use. Keep on ice once thawed. Thawed Releasing Buffer can be refrozen once.

CELLectionTM Dynabeads washing procedure

The CELLectionTM beads should always be washed before use. The washing procedure is achieved using a Dynal MPC magnet.

1. Resuspend the CELLectionTM beads thoroughly in the vial.
2. Transfer the desired amount of CELLectionTM beads to a test tube.
3. Place the test tube on a Dynal MPC and pipette off the fluid.
4. Remove the vial from Dynal MPC, add 1-2 ml of washing buffer (PBS / 0.1% BSA / 0.6% Na citrate) and resuspend.
5. Place the tube in the Dynal MPC, pipette off the fluid and resuspend the washed CELLectionTM beads in a volume of washing buffer equal to that originally pipetted from the vial.

NOTE: During incubation and separation procedures, it is important to keep the cell suspension and buffer cold (2-8°C) to prevent attachment of phagocytic cells to CELLectionTM Dynabeads.

PROTOCOL B

Tumour cell enrichment from bone marrow and analysis with EPiMET® Enrich
Starting materials (not including controls):
1-2 ml bone marrow
250 µl CELLectionTM beads and 4 µl reconstituted Releasing Buffer
1 large spot cytospin for staining with EPiMET® Enrich
DNase treatment of bone marrow (optimal)(2) (not doing this part)
Using 1-2 ml bone marrow (107 - 108 cells):

1. To bone marrow sample, add cold buffer to increase volume (Hanks or PBS / 0.1% BSA)
2. Centrifuge sample for 10 minutes, 600 - 800 xg at room temperature.
3. Remove supernatant and resuspend cells in 10 ml PBS / 0.1% BSA / 0.5 mM MgCl2 / 1 mM CaCl2 / 100 U per ml DNase. (DNase for this step is not included in the kit).
4. Incubate cells for 30 minutes at room temperature using an apparatus that allows both gentle tilting and rotation.
5. Centrifuge cell suspension for 10 minutes, 600 - 800 xg at room temperature.
6. Remove supernatant completely and resuspend cell pellet in 10 ml PBS / 0.1% BSA / 0.5 mM MgCl2 / 1 mM CaCl2 / 0.6% Na citrate.
7. Repeat steps 5 and 6 at least two times to remove the DNase.
8. Resuspend cells in 5 ml cold PBS / 0.1% BSA / 0.6% Na citrate / 0.1% casein (pH 7.0 - 7.2). Then add EDTA to a final concentration of 1 mM. Continue with step 4 below.

Positive isolation of tumour cells from bone marrow

1. Wash 1-2 ml bone marrow by adding equal volume PBS / 0.1% BSA / 0.6 Na citrate (DNase treatment of the bone marrow sample as described above is an alternative to the steps 1-3 described here).
2. Centrifuge at 600 - 800 xg for 10 minutes. Pipette off supernatant.
3. Resuspend to 5 ml with PBS / 0.1% BSA / 0.6% Na citrate.
4. Add 250 µl washed CELLectionTM beads to 5 ml diluted bone marrow.
5. Mix bone marrow and beads thoroughly and incubate on a device allowing both tilting and rotation at 2-8°C for 30 minutes.
6. Place in a magnet (Dynal MPC) for 3 minutes before supernatant is removed by pipetting.
7. Resuspend bead/cell rosettes in 1-2 ml PBS / 0.1% BSA and transfer to a new tube such as an Eppendorf (use washing buffer with no citrate).
8. Place the tube in a magnet (Dynal MPC) for 2 minutes and pipette off the fluid.
9. Repeat washing procedure 3 times before resuspending washed rosetted cells in 200 µl preheated (37°C) RPMI / 1% FCS.
10. Proceed with keeping beads attached (Protocol 1) or releasing beads (Protocol 2). See notes above for optimal cell release.

Protocol 1: Positive Isolation of Tumor Cells Keeping Beads Attached (no bead removal)

1. Add the washed 5 ul of Dynabeads Epithelial Cell resuspended in Washing solution, to each 1 ml cell sample and leave on roller for 30 min at 2-8 degrees C for rosetting to occur. Put roller in the fridge or do this tube wall. Invert the magnet with the tubes after appox. 1 min in order to collect beads left in the cap of the tubes.
2. Remove the supernatant carefully by pipetting. Be sure not to disturb the bead pellet. Save the supernatant and count the cells to see how many bone marrow cells you got in the separation. Compare to
3. Add 800 ul cold Washing solution. Remove the tubes from the magnet and resuspend the rosettes
4. Place the eppendorf tube on the magnet 2-3 min. Remove the supernatant without disturbing the bead
5. Place in a magnet (Dynal MPC) for 3 minutes before supernatant is removed by pipetting.
6. Remove supernatant and resuspend bead/cell rosettes in 800 ul PBS / 0.1% BSA and transfer to a new tube such as an Eppendorf (use washing buffer with no citrate).
7. Place the tube in a magnet (Dynal MPC) for 2 minutes and pipette off the fluid.
8. Repeat washing procedure 2 times before resuspending washed rosetted cells in 50 µl preheated (37°C) RPMI / 1% FCS.

Protocol 2: Removal of Dynabeads, Releasing of positively isolated cells

1. Add 200 U (4 µl) Releasing Buffer to the rosetted beads/cells which are resuspended in preheated buffer.
2. Mix gently and incubate for 15 minutes on a mixing device at room temperature.
3. Take off cap and pipette vigorously with a 150 µl pipette at least 8 times. Failure to pipette the sample will affect cell yield.
4. Place in a magnet (Dynal MPC) and transfer by pipetting the released cells into a new Eppendorf tube precoated with RPMI / 10% FCS.
5. Wash out remaining cells from the bead fraction by adding 200 µl RPMI / 1% FCS to the beads. Pipette at least 5 times and place in a magnet (Dynal MPC).
6. Transfer the supernatant to the new tube with the released cells. Released cells are in a final volume of 400 µl RPMI / 1% FCS.
7. Then add 200 ul of 1% LMP agarose in EF to the 100 ul of cells and RPMI/1% FCS.

SOP 8. Protocol for Processing Bone Marrow Samples with Ficol Paque (RT)

From Amersham manual

Processing:

Sterile Techniques are employed.

Materials Needed:

Solutions: PBS (RT), Ficol Paque (RT) Amersham, Medium 199 + 10% FBS (4 C)

Others: 50 ml tubes, 15 ml tubes, and pipettes

Preparations:

1. Dilute Bone Marrow with 2 ml of PBS.
2. Invert Ficoll Paque Plus bottle many times to ensure through mixing.
3. Snap off polypropylene cap and insert syringe through the septum. Invert the bottle and withdraw with.3 ml Ficoll Paque in a centrifuge tube.
4. Carefully layer the diluted Bone marrow sample on the Ficol Paque Plus.
5. Centrifuge for 400xg for 30-40 minutes at 18-28 C, no brake.
6. Draw off the upper plasma layer using a clean Pasteur pipette, leaving the mononuclear cell layer undisturbed at the interface.
7. Transfer the mononuclear cell layer only into a clean 15 ml tube and fill with 6 ml of PBS in the tube. Suspend the cells by gently drawing then in and out of a Pasteur pipette.
8. Centrifuge at 60-100xg for 10 minutes, discard supernatant, resuspend and repeat.
9. Resuspend mononuclear cells in 1 ml RPMI or the medium appropriate to the application, mix well and count cells.

SOP 9. Standard Operating Procedure for Original Comet Assay Method Using Smaller Area On Slide - Preparations Before Experiment

a. Equipment and Materials

Dynal Magnet Particle Concentrator (Dynal MPC)
Water bath
Dynal Mixing device the allows both tilting and rotation of test tubes
Hemacytometer
Microscope
Incubator
Mettler H54AR balance
Electrophoresis unit with buffer
Laser Scanning Cytometer (LSC)
Flourescent Microscope
Coldroom
Frozen bone marrow samples from patients with metastatic breast cancer to the bone
Dynabeads Epithelial Enrich kit (not releasing the beads): Suspension containing 4x108 beads/ml in phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02 % sodium aside (NaN3).
Chemotherapeutic drugs: BCNU, Cisplatin, and Cyclophosphamide

Antibodies

Alexa Fluor 647 Monoclonal Atibody Labeling Kit (Molecular Probes A-20186)

Kit Contents:

· Alexa Fluor 647 reactive dye (Component A), five vials
· Sodium bicarbonate (MW = 84, Component B), ~84 mg
· Purification resin (Component C), ~10 mL of 30,000 MW size–exclusion resin in phosphate-buffered saline (PBS), pH 7.2, plus 2 mM sodium azide
· Spin columns (Component D), five columns
· Collection tubes (Component E), five 2 mL tubes

Glutathione S-Transferase (GST) Pi Rabbit Antibody and 488 Goat Anti-Rabbit (GAR) Secondary Antibody
Purified Mouse Anti-Human Cytokeratin 18 Monoclonal Antibody (BD PharMingen 550508)

b. Preparations that need to be done before the day of the experiment:

i. Protocol for the advanced preparation of the Alexa Fluor 647 Monoclonal Antibody Labeling Kit (A-20186) the uses the Purified Mouse Anti-Human Cytokeratin 18 Monoclonal Antibody

Conjugation Reaction

1.1) Prepare a 1 M solution of sodium bicarbonate by adding 1 ml of deionized water (dH2O) to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved. The bicarbonate solution, which will have a pH ~8.3, can be stored at 4°C for up to two weeks.

1.2) If the antibody to be labeled has a concentration of ³1 mg/ml and is in an appropriate buffer (see Protein Preparation), dilute it to 1 mg/ml and then add one-tenth volume of 1 M sodium bicarbonate buffer (prepared in step 1.1). If the protein is a powder lyophilized from an appropriate buffer, prepare a 1 mg/mL solution of the antibody by adding an appropriate amount of 0.1 M sodium bicarbonate buffer to the protein. Prepare 0.1 M sodium bicarbonate buffer by diluting the 1 M solution 10-fold with dH20. Bicarbonate, pH ~8.3, is added to raise the pH of the reaction mixture, since succinimidyl esters react efficiently at pH 7.5–8.5.

1.3) Transfer 100 ul of the protein solution (from step 1.2) to the vial of reactive dye. Cap the vial and gently invert it a few times to fully dissolve the dye. Violent agitation of the protein solution can result in protein denaturation. To visually confirm that the dye has fully dissolved, it may help to peel the label off the vial of reactive dye.

1.4) Incubate the solution for 1 hour at room temperature. Every 10–15 minutes, gently invert the vial several times in order to mix the two reactants and increase the labeling efficiency.

During the incubation period, proceed to steps 2.1–2.4 in order to prepare a spin column for the purification of the labeled protein. This will take about 15 miinutes.

Purification of the Labeled Protein

2.1) Place a spin column in a 13 × 100 mm glass tube.

2.2) Stir the purification resin (Component C), then add 1.0 mL of the suspension into the column and allow it to settle.

2.3) Continue to add more of the suspension until the bed volume is ~1.5 ml. Allow the column buffer to drain from the column by gravity. Initially, some pressure may be required to cause the first few drops of buffer to elute.

2.4) Place the spin column in one of the provided collection tubes and centrifuge the column for 3 minutes at 1100 × g using a swinging bucket rotor. Discard the buffer, but save the collection tube. The spin column is now ready for purifying the conjugated antibody. A fixed angle rotor will suffice if a swinging bucket rotor is not available.

2.5) Load the 100 ul reaction volume (from step 1.4) dropwise onto the center of the spin column. Allow the solution to absorb into the gel bed.

2.6) Place the spin column into the empty collection tube and centrifuge for 5 minutes at 1100 × g.

2.7) After centrifugation, the collection tube will contain labeled protein in approximately 100 ul of PBS, pH 7.2, with 2 mM sodium azide; free dye will

Determination of Degree of Labeling

3.1) Dilute a small amount of the purified conjugate into PBS or other suitable buffer and measure the absorbance in a cuvette with a 1 cm pathlength at both 280 nm (A 280) and 650 nm (A 650).

3.2) Calculate the concentration of protein in the sample:

Protein concentration (M) = [A280 - (A650 * 0.03)] * dilution factor/203,000
where 203,000 is the molar extinction coefficient in cm -1 M -1
of a typical IgG at 280 nm and 0.03 is a correction factor for the
fluorophore's contribution to the absorbance at 280 nm.

3.3) Calculate the degree of labeling:

Moles dye per mole protein = A650 * dilution factor/(239,000 * protein concentration (M)
where 239,000 cm -1 M -1 is the approximate molar extinction coef-ficient of the Alexa Fluor 647 dye at 650 nm. For IgGs, we find that labeling with 3–7 moles of Alexa Fluor 647 dye per mole of antibody is optimal.

Storage of Conjugates

Store the labeled protein at 4°C, protected from light. If the final concentration of purified protein conjugate is less than 1 mg/ml, add BSA or other stabilizing protein at 1–10 mg/ml. In the presence of 2 mM sodium azide, the conjugate should be stable at 4°C for several months. For longer storage, divide the conjugate into small aliquots and freeze at -20°C. AVOID REPEATED FREEZING AND THAWING. PROTECT FROM LIGHT.

ii Making Up Buffers/Solutions

Growth medium (EF) and FBS/EF

Trypan Blue for cell counts

Solvent for drug: DMSO

Washing solution for Dynalbeads (not removing the beads): PBS with 1% (w/v) fetal calf serum (FCS)/0.6% NaCitrate.

All reagents should be analytical grade. The fetal calf serum should be inactivated for 30 min at 56 degrees C and tested for cytotoxicity before use.

(PBS+0.1%BSA+Cd+0.1%Saponin)

(PBS+0.1%BSA+0.1%Saponin) Combine: 500 ml PBS, 0.5 ml BSA/EF (10%), 0.5 g Saponin into a 500 ml bottle with a magnetic stirring bar. Place on stirrer for 30 minutes, store in refrigerator.

(PBS+BSA+0.3%Saponin) Combine: 500 ml PBS, 0.5 ml BSA/EF (10%), 1.5 g Saponin into a 500 ml bottle with a magnetic stirring bar. Place on stirrer for 30 minutes, store in refrigerator.

1% LMP Agarose in EF

SyBR Green Stain:

To make SYBR green dilution: Mix up 1 ml of TE buffer and then add 1ul of Rnase per ml of TE buffer (1ul) and mix with 1 ul SYBR green stock.

Antifade (Prolong)

iii. Comet Assay Supplies and Solutions

In the comet assay there are many steps to consider.

The assay can be very consuming. Proper planning can help make the assay smooth and predictable.

One should first plan out the treatment procedure and slides for set up.
Solutions such as lysis buffer and neutralization buffer should be set ahead of time.
Slides should be labeled and dipped in the 1% agarose as described below..
Slides should always be handled with clean gloves and when set on the bench top be set on clean, Kim wipe.
The whole assay should be handled carefully with clean gloves which are changed often.
Dipped slides should be stored in a clean slide box (not a dusty one).
With the set up in place the assay can be run.

Preparations for the Comet Assay:

clean glassware: one liter flasks, slide holders

washed and autoclaved

dipped slides: microscope slides dipped in 1% normal melting point agarose in PBS
using an autoclaved beaker, place 100 ml of PBS and add one gram of normal melting point agarose
microwave for one minute, after agarose has dissolved, pour into clean 50 ml centrifuge tube
dip microscope slide past the frosted area
wipe the underside with small Kim wipe and place on large, clean Kim wipe
allow to dry completely before storing in a microscope box

sterile 1.5 ml microfuge tubes
place many microfuge tubes in a container special for autoclaving materials
autoclave

0.5% low melting point agarose in bottles
using an autoclaved beaker, place 100 ml of PBS
add one gram of normal melting point agarose
microwave for one minute
after agarose has dissolved, pour into clean sterile scintillation vials

sterile water: treated with DEPC over night
Makes 100 ml of DEPC H20
Add 125 ul Diethyl Pyrocarbonate (DEP or DEPC) to 100 ml of purified H20
- Place on stir-plate with magnetic stir-bar and mix for 1 hour
- Autoclave for 20 minutes, remove stir-bar
- Store at room temperature

EDTA Solution
Makes 100 ml of 0.5M EDTA at pH 8.0
- Dissolve 18.6 grams EDTA in 100 ml DH20. Use NaOH to adjust pH
- Store at room temperature

one liter alkali lysis solution
the day before: ingredients per 1000 ml
2.5 M NaCl 146.1 g
100 mM EDTA (disodium) 37.2 g
10 mM Tris-base 1.21 g
pH to 10 with NaOH 6-7 g
1% sodium lauryl Sarcodine 10 g
Bring this solution up to 890 ml using sterile DEPC treated water, store at room temperature.
add the day of:
1% Triton X-100 10 ml
10% DMSO 100 ml
**Remember to refrigerate prior to slide addition for 30-60 minutes*
**Store unused portion in cold room and keep at constant stir*

neutralization buffer 0.4 M Tris, pH 7.5
63.04 g Tris (acid), fill to 1000 ml with dH20
Set pH to 7.5 using NaOH, HCL
Store in refrigerator

electrophoresis buffer
Add 5 ml of 10N NaOH and 2 ml of 0.5M EDTA to a final volume of 1000 ml in DEPC treated water.
Stock solution for 10N NaOH - 40 gram NaOH to 100 ml DEPC treated water.

For an assay run under neutral pH conditions, the lysis buffer and electrophoresis buffers are different. All other parts of the procedure are the same.

one liter neutral lysis solution
30 mM EDTA 60 ml 0.5 M EDTA
0.5% Sodium Dodecyl Sulfate 5 g
bring to pH 8.3

one liter electrophoresis buffer
Use TBE (Tris boric EDTA)
Ready weighed powder available from USB (a division of Amersham Int'l)
Dissolve the powder in 100 to 150 ml of sterile water DEPC treated.
After material is dissolved, bring the total volume up to 200 ml
This is a 10X solution of TBE buffer. Dilute to 1X for use.
TBE is
90 mM Tris base 10.8g/l
90 mM boric acid 5.6 g/l
2 mM EDTA 0.74 g/l
pH 8.0

Other materials
Serological pipettes
spatula
cuvette
Gloves

Standard Operating Procedure for Original Comet Method Using Smaller Areas

Procedure:

Prepare an experimental setup data table. Include the patient sample number, planned drug exposures, assign each tube a number, and label the bone marrow slides according to the date and the corresponding tube number.

c. Preparation of Bone Marrow and MCF-7 WT Cell Mixture for Spiking Experiments:

1. Thaw bone marrow sample quickly in a water bath and add to a 15 ml centrifuge tube. Resuspend the cells in 4 ml EF medium and centrifuge at 310xg for 5 minutes. Remove the supernatant and resuspend cells in 1 ml EF. Count the cells. Always be careful never to foam the suspensions. Hold on ice.

2. To prepare the MCF-7 WT cells first warm up media to 37 C in 3/4 full water bath: EF, 5% FBS/EF, FBS, and 0.05% Trypsin-EDTA. Then take out a T25 flask with the growing cells in it out of the incubator and pour away old medium from the flask in to beaker with bleach (waste). Rinse with 1 ml of Trypsin and pour into waste beaker, then repeat. Add 1 ml Trypsin to flask an incubate at 37 C for 7-10 minutes. Add 4 ml EF and wash bottom of flask. Pipette up and down until you get an even cell suspension. Transfer suspension to a 15 ml centrifuge tube containing 1 ml FBS and centrifuge for 310xg for 5 minutes. Remove the supernatant and resuspend the cells in 1 ml EF. Count the cells.

3. Cell count is determined by Trypan blue exclusion by the following:

90 ul Trypan blue
10 ul cell suspension
Mix by pipetting in a well and count on a hemocytometer
Count the four side squares and take the average of all of these squares. Then multiply the average x 10 x 10e4.

4. After Counting, make up the MCF-7 WT:BM mixes in 1.5 ml centrifuge tubes:

Wt:BM mix:
MCF-7 WT (starting with cell concentration of 1,000,000 cells/ml)
Do 1:10 dilution with EF (100,000 cells/100 ul)
Take 100 ul and mix with 900 ul of BM with the concentration of 10,000,000 cells/ml in 1 ml tube (10,000 MF-7 WT:9,000,000 BM)
Cell Ratio 1:900
Then take 100 ul into new tube (1000 MCF-7 wt:900,000 BM cells)

Preparation of the Patient Bone Marrow Sample:

1. Preparation of bone marrow sample for the reception of the drug: Thaw bone marrow sample quickly in a water bath and then centrifuge at 1000xg for 10 min. Resuspend cells in one ml RPMI 1640 growth medium and set aside a aliquot of the sample to estimate the cell count. Wait to count the cells, count the cells during the incubation time with the drug.by using a hemacytometer. Always be careful never to foam the suspensions. Hold on ice.

2. Cell count is determined by Trypan blue exclusion by the following:

90 ul Trypan blue
10 ul cell suspension
Mix by pipetting in a well and let stand for 5 min. and count on a hemocytometer
Count the four side squares and take the average of all of these squares. Then multiply the average x 10 x 10e4.

3. Now determine the volume of cell suspension needed to put the necessary number of cells into the assay system or you can just alliquot the sample into the separate tubes.
4. Bring up the volume of each assay tube. Noting volume of cell suspension in each assay tube and that 1% of the final volume is the drug in it's solvent: The rest of the volume should be filled with growth medium. Typically the volume is one ml.

d. Procedure for Determining Drug Treatment Dosage Using Mettler H54AR:

Planned drug exposures: 0uM 3uM 10uM 30uM 100uM

1. The Drug Calculations:

Weigh the proper amount of drug needed:

a. Weigh vials
b. Adjust coarse adjustment weight using "1/2"
c. Fine tune on "1" (bars between teeth)
d. Remove static (when necessary) using static gun
e. Take vial to chemical exhaust hood
f. Using a spatula take and add small amount to respective tube
g. Close tube and weigh on Mettler; weigh on "1"
h. Get weight in miligrams and convert into micrograms. To find the amount of DMSO solvent that needs to be added to make a 1 M standard use the formula: Weight in g/Molecular Weight of BCNU = ml solvent (DMSO) needed for 1M

2. Set up the dilutions and label all of the tubes according to the BCNU Rx chart that is attached.

To do serial dilutions, first label all vials:B1M, B100, B100, B10, B10, B1, and DMSO only (control)

Pipette Required Solvent into each vial: 270, 350, 270, 350, 270, and 350
Use B1M to make first dillution 30ul
Use B100 to make next two dilutions using 150ul and 30ul of stock solution
Use B10 to make next two dilutions using 150ul and 30ul of stock solution
Use B1 for last Dilution with 150ul of stock solution
This will give you the new mM concentration now you can relabel your vials to correspond with these new concentrations. (B100, B30, B10, B3, B1, B0.3, and DMSO only (control)) Once the drug treatment stocks are made up they can be stored in the freezer for use in later experiments. When the solvent is added to the drug and mixed, action from there must be quick.

e. Adding the drug treatment stocks to the Bone Marrow Sample:

3. Have ready the assay tubes with cells and RPMI 1640 growth medium ready for the reception of the drug. Now you add the drug treatment stock solutions to the corresponding tubes so that the drug is never 1% more than the total volume in the tube because the final concentration of the dilution is 100 fold of these drug treatment stocks. Considering this, the treatment stock solution must be 100 times more than the desired concentration (multiply by 100 to the the molar concentration) Example, when 10ul is added to the assay tube the final concentration of the treatment drug in the total of 1000 ul will be the desired level.

4. After the drug is added to each assay tube, the tubes should be quickly inverted in order not to create a gradient where the cells at the surface are exposed to drug for longer than the rest of the cells throughout. After all tubes have been inverted invert tubes again. Work in the dark in order to protect the cells from light. Always be careful never to foam the suspensions. Place tubes in a metal cube and Incubate assay tubes at 37 degrees C for two hours in an incubator. After treatment the tubes should be spun down at 210xg for 10 minutes at 4 degrees C.

5. Spin down the cels at 1000 rpm for 10 minutes and then dilute the cell suspension to a concentration of 1-2x107 cells/ml in washing solution (PBS/1% FCS/0.6% NaCitrate) by adding 1 ml into the tubes where beads will be added. For the tubes with no beads (just bone marrow samples with no tumor cells) proceed onto the antibody staining procedure ans spin down tubes at 210xg for 10 minutes instead of using the magnet each time..

f. PROTOCOL: Adding the dynal beads to the cell suspensions after drug incubation:

Prepare the magnetic beads for use. The Dynabeads Epithelial Enrich should be washed before use. The washing procedure is achieved using the Dynal MPC. Use 5 ul beads per ml cell suspension. Pool the beads needed for all samples in an eppendorf tube.

a. Resuspend the Dynabeads Epithelial Enrich thoroughly in the vial.

b. Transfer the desired amount of Dynabeads Epithelial Enrich to a tube.

c. Place the tube on a Dynal MPC and pipette off the fluid.

d. Remove the vial from Dynal MPC; add 1-2 ml of washing buffer and resuspend.

e. Repeat step c., and resuspend the washed Dynabeads in a volume of washing buffer equal to that originally pipetted from the vial (5ul).

Positive Isolation of tumor cells from bone marrow if not releasing the Dynal beads:

1. Add the washed 5 ul of Dynabeads Epithelial Cell resuspended in Washing solution, to each 1 ml cell sample and leave on roller for 30 min at 2-8 degrees C for rosetting to occur. Put roller in the fridge or do this step in a coldroom.

2. Place the tube in the magnet for 2-3 min at 2-8 degrees C, for the bead-cell complexes to migrate to the tube wall. Invert the magnet with the tubes after appox. 1 min in order to collect beads left in the cap of the tubes.

3. Remove the supernatant carefully by pipetting. Be sure not to disturb the bead pellet. Save the supernatant and count the cells to see how many bone marrow cells you got in the separation. Compare to the first cell count that was done in the beginning of the experiement.

4. Add 800 ul cold Washing solution. Remove the tubes from the magnet and resuspend the rosettes carefully.

5. Place the eppendorf tube on the magnet 2-3 min. Remove the supernatant without disturbing the bead pellet.

g. Immunohistochemistry with Antibodies:

Note: Turn on the slide warmer and put the Predipped agarose slides and pipette tips on it to warm up before adding the samples to the slides.

1. Resuspend the bead pellet in 100 ul (PBS+0.1%BSA+**Cd**+0.1%Saponin) and add 10 ul Superblock and incubate on ice for 5 minutes. Then place on the magnet for 2-3 minutes. Remove the supernatant while the tube is in the magnet.

2. Resuspend in 100 ul (PBS+BSA+0.3%Saponin).

3. Add 10 ul of the Glutathione S-Transferase Pi antibody to the tubes and then add 2 ul of the already prepared Cytokeratin antibody kit (preparation is mentioned above in materials) to the bead pellet and incubate tubes with both antibodies in them 1 hour on ice.

4. Wash cells with 100 ul (PBS+BSA+0.1%Saponin), place in magent for 2-3 minutes and then pipette out the supernatant and pick up in 100 ul (PBS+BSA+0.3%Saponin).

5. Add 2 ul secondary antibody (488 GAR secondary) and incubate 1 hour on ice.

6. Wash cells with 100 ul (PBS+BSA+0.1%Saponin), put tube in the magnet for 2-3 minutes and then pipette out the supernatant and then resuspend the cells and beads in 30 ul (PBS+BSA). Do each tube one at a time and be very carefull to avoid bubbles when resuspending the cells.

h. The Comet Assay:

Slide Preparation for the Comet Assay

1. First layer:

Warm up a tube of 1% LMP Agarose in EF in a beaker half filled with water in a microwave until it is in liquid form. *** Remember to take the cap off the agarose before heating because it will explode. Then add the agarose tube into a 50 degree C water bath for about 15 minutes. Have all of the slides set out with cover slips next to it before the agarose is added to the cell suspensions. Hold the assay tubes on in the water right before adding a appropriate volume of agarose. Work quickly so that the agarose does not gel before it is even on the slide.

2. Add 60 ul of 1% LMP agarose in EF to the 30 ul of cells and washer to one tube at a time. After adding the agarose, take 30 ul of agarose and cell mixture and add PER slide to be layered. Quickly drop the coverslip over the slide and push out the air bubbles with your fingers. Have a square ice bucket filled and level with ice. The slides should be on a metal tray on the ice for a minimum of three minutes.

3. Second layer:

of the slide and remove the slip. Take up 30 ul of clean 1% agarose (no cells) and carefully place another cover slip on the slide and push the agarose up with your fingers. Again, place the slides on ice for at least three minutes. Locate the cells with flourescent microscope/LSC. Scan slides using two lasers (Green fluorescent Argon laser for GST pi and Long Red fluorescent HeNe Laser for Cytokeratin).

4. After, remove the cover slips and place the slides in slide holding glass containers and pour in the completed lysis solution chilled. Do this carefully and from the side so that the layers of agarose on the slides are not washed away. These slides should set in the lysis solution overnight or for about 3 hours.

The next day:

5. Prepare the electrophoresis buffer and pour into the electrophoresis chamber. Attach the hosing so that the cooling unit is connected to the electrophoresis unit. Set the cooling unit to less than 10 degrees C. Set the slides onto the platform of the electrophoresis chamber. Set them all in the same direction. Let the slides set in the electrophoresis buffer with the cooling unit on cold for 20 minutes to denature the DNA. (This is not necessary for the neutral assay)

6. Run the power supply at 25 volts, 300 mA for 30 minutes. Amperage is adjusted by altering the volume of the buffer brings the amperage to 300 mA. It will be necessary to pour in about 700 ml of buffer and run the power supply. If the amperage is high, the power supply should be turned off and some volume of the buffer removed. If the amperage is too low then buffer should be added. After 30 minutes, turn off the power supply completely.

Use Extreme Caution! Make sure power supply is off and leads are safely away from liquids before manipulating the buffer volume!

7. Submerge slides in neutralization buffer for 5 minutes, rinse in dH20 for 5 minutes, and fix in ethanol for 5 minutes. (This is best done using the glass slide holder and the individual glass containers which are in the coldroom). Airdry the slides for at least 30 minutes. Slides can be stored indefinitely at this point. After all the slides are dry then stain with SYBER Green.

8. Stain with SYBR green: Cover slides with 200 ul of SYBR green dilution. (To make SYBR green dilution: Mix up 1 ml of TE buffer and then add 1ul of Rnase per ml of TE buffer (1ul) and mix with 1 ul SYBR green stock per ml of TE buffer.). Then incubate for 10 minutes at 37 degrees C.

9. Rinse slides 3X by submerging them in 50 ml H20

10. Cover with 100 ul antifade (Prolong) and coverslip. For long storage seal sides of the coverslip with clear nailpolish. Slides may be stored in the dark for a few days.

11. Scan the slides on the LSC to find the cells and score the comets. Store slides in the dark.

12. After Comet Assay treatment, the slides were stained and then rescanned with either the Green Argon laser for the DNA stain SYBR Green I (Molecular Probes S-7567) or the Long Red HeNe laser for the monomeric cyanine nucleic acid stain To-Pro-3 (Molecular Probes T-3605).

I. Directions for Data Analysis

1. Gate out the junk that is not cells from the first scan.
2. Gate out the cells from the second scan that have only one FISH spot in the comet.
3. A dose response analysis can bo done on the comet scan data. On the Wincyte software, make a ratio histogram of Integral /FISH and set up two different regions according to the control of the experiment. The gating should be set so the undamaged region contains 95% of cells and damaged region the remaining 5%of cells. After setting up the control, work back to get the percentage of damage in all other doses. If there was a successful dose response then the % of damaged cells should increase as the treatment dose increases.
4. Merge the first and second scan together, using the gated versions of second scan as the merge one file and the gated version of the first scan for the merge two file. Green I = SYBR Green and Green II = GST Pi
5. After merging the files, save cells in region to get the exact matching cells for each file. Save as FCS. File and choose the option Text Data Dump to save as a text file in the Wincyte program.
6. Copy the text files for each sample into the Comet Datatbase file on the active drive and make a separate file for each patient.
7. To format the text file, cut off header using the shift key, save as and assign the file a new name by adding fmt for each formatted file.
8. Import the text file into into Microsoft Access one at a time and make a new database per patient, each sample a new table.
9. Make a copy of each table with f1 added at the end, to designate the FISH head table.
10. For the main table save only the numbered line, use sort and delete the blank and F1 lines. For the F1 tables use backward sort to get rid of all of the blank and numberd lines, keep only the F1 lines.
11. Add identifier variables to each table including, patient number, time of the sample, sample number, and the drug dose. The coding for the time of sample: BI (Before Induction) = 1, AI1 (After Induction 1, Eval 1) = 2, AI2 (After Induction 2, Eval 2) = 3, and AR (After Relapse) = 4.
12. Change the labels in the design of the main table by adding cell for the whole cell data or head for just teh FISH data and SYBR Green and GST Pi to designate the correct labeling in the correct order, remember that SYBR Green comes first because it is Green I and GST is last because it is Green II.
13. Make a query with all of the equations for calculating the Integral Multiples and the Tail Moments using the different variables stated below.
Formulas: Add in the Appropriate Antibody, SYBR Green or GST Pi.
Integral Multiple = ((Integralcell-Integralhead)/Integralcell)
Diff in SQRT of Area = SQRT(Areacell)-SQRT(areahead)
SQRT of diff in Area = SQRT (Areacell-areahead)
Diff in X Value = Xcell-Xhead
Diff in Y Value = Ycell-Yhead
The Calculated Distance Value (Z) = square root(Diff in X^2 + Dif in Y^2)
For the Tail Moment Calculations multiply the integral multiple by next 4 varables, Diff in SQRT of Area, SQRT of diff in Area, Diff in X Value, and Z value.
TM Diff in SQRT of Area = Integral Multiple*Diff in SQRT of Area
TM SQRT of diff in Area = Integral Multiple*SQRT of diff in Area
TM Diff in X Value = Integral Multiple*Diff in X Value
TM Z Value = Integral Multiple*Z Value
14. Merge the two separate tables to make one big table that is only one line with all of the information.
15. Merge all the samples for one patient together in one table to include all of the info for that patient.

16. Compare the different tail moments for each sample and take the average in Excel, look at the trend in one time sample and then compare each different time sample with each other to see if the experiments followed the original hypothesis.

17. Make graphs using the Average Tail Moment Calculated Z Value vs. Drug Dose.

C. Reportable Outcomes

Land, S.J., Bacsó, Zs., Klein, J., Eliason, J.F., and Everson R.B. Quantitative methods for examining the drug resistance phenotype of micrometastatic cancer cells in bone marrow: DNA damage in response to in vitro drug treatment. *Proceedings of the American Association for Cancer Research* 40:403,1999

Toset, A.W., Everson, R.B.: Measurement of DNA repair in BRCA1 and BRCA2 deficient human cell lines with the Single Cell Gel Electrophoresis (SCGE) assay. *Proceedings of the American Association for Cancer Research* 41: 335, 2000

Everson, R.B., Land, S.J., Eliason, J.F., Klein, J.L., and Baynes R.D. Transient resistance to high-dose chemotherapy: Evaluation of the use of DNA-damage assays to optimize treatment schedule. *Proceedings of the Department of Defense Breast Cancer Research Program Meeting: Era of Hope* Vol. II: 690, 2000

Bacso, Z., Everson, R. B., and Eliason, J. F. The DNA of annexin V-binding apoptotic cells is highly fragmented. *Cancer Res*, 60: 4623-4628., 2000.

ABSTRACT

Quantitative methods for examining the drug resistance phenotype of micrometastatic cancer cells in bone marrow: DNA damage in response to *in vitro* drug treatment. Land, S.J., Bacsó, Zs., Klein, J., Eliason, J.F., and Everson R.B. Karmanos Cancer Inst., Wayne State University, Detroit, MI.

To examine the sensitivity of the cancer cells in patient bone marrow samples to the drugs used for high dose therapy, we have used the single cell gel electrophoresis (comet) assay for measuring DNA damage. Studies were performed with MCF7 breast cancer cells, which were incubated at 37°C with various concentrations of 4-OH cyclophosphamide, cisplatin or BCNU. Levels of DNA damage as measured by Olive tail moment (product of DNA fluorescence in the tail and tail length) increased with increasing concentrations of each drug. To determine if these measurements could be performed in the presence of human bone marrow cells, mixtures of MCF7 cells with normal marrow cells were prepared and treated with BCNU. The breast cancer cells were enriched from the mixtures using a panel of 3 murine monoclonal antibodies that recognize antigens on human breast cancer cells and magnetic beads conjugated with antibodies against the primary antibodies. The rosetted cells were enriched by magnetic sorting and analyzed using the comet assay. Only those cells associated with 3 or more beads were scored. Staining of beaded cells with antibodies against cytokeratins indicated that they were cytokeratin positive. The results for rosetted cells demonstrated similar levels of DNA damage compared to MCF7 cells assayed as a pure population. This assay has also been performed with metastatic cells isolated from patient bone marrow samples. This approach will provide a measure of drug sensitivities of malignant cells in bone marrow.

TRANSIENT RESISTANCE TO HIGH-DOSE CHEMOTHERAPY. EVALUATION OF USE OF DNA-DAMAGE ASSAYS TO OPTIMIZE TREATMENT SCHEDULE

Drs. Richard B. Everson, Susan J. Land, James F. Eliason, Jared L. Klein, and Roy D. Baynes

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For drugs that interact with DNA, measures of DNA damage can assess the intracellular availability of active drug at a critical molecular target. Measurements of DNA damage should reflect the integrated effect of all resistance factors, including both recognized mechanisms and uncharacterized mechanisms. Thus, molecular measures of DNA damage could provide an important tool for elucidating the time course of complex changes in resistance factors.

Motivated by a recent clinical trial that demonstrated better survival when the interval between induction chemotherapy and high dose therapy was prolonged, this project is using measures of DNA damage in patient blood cells to determine whether induction chemotherapy causes transient changes in resistance. We are using the single cell gel electrophoresis (comet) assay and assays for chemical modifications to DNA. Prior to analysis of clinical specimens, studies of the capability of these methods were investigated with MCF7 breast cancer cells and human lymphocytes and bone marrow cells. The cells were incubated at 37°C with various concentrations of 4-OH cyclophosphamide, cisplatin, or BCNU.

Each agent produced DNA damage that can be measured in a dose dependent manner in the single cell gel assay. Increases were large: for example, mean levels for damage were 4.3, 10.2, and 102 with 0, 10, and 100 uM BCNU, respectively. At low doses, each agent causes a similar pattern of breakage, while at high doses their pattern of damage could be distinguished. Cisplatin contracted the cells, consistent with its ability to cause DNA crosslinks. BCNU caused a pattern suggesting diffusion of relatively constant molecular weight fragments prior to electrophoresis and displacement of those fragments during electrophoresis, a pattern shown to indicate apoptosis. 4-OH cyclophosphamide caused broad diffusion of the DNA, a pattern previously linked with necrosis.

Both lymphocytes and bone marrow cells from patients can be routinely analyzed, allowing study of changes in sensitivity in multiple tissues. It is feasible to use the procedure to study whether induction therapy has a transient effect on resistance to high dose therapy; these studies are underway.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8352 supported this work.

Measurement of DNA repair in BRCA1 and BRCA2 deficient human cell lines with the single cell gel electrophoresis (SCGE) assay

Toset, A.W. and Everson R.B., Karmanos Cancer Inst., Wayne State University, Detroit, MI

Recent investigations link the function of BRCA1 and BRCA2 with deficiencies in DNA repair, including repair of oxidative damage, radiation damage, and damage from chemotherapeutic agents that cause double strand breaks. Because of its ability to analyze small numbers of cells from biopsy samples, use of the SCGE to measure these deficiencies would facilitate translational research by allowing parallel studies of cell lines and other experimental systems and clinical specimens. To ascertain the effectiveness of the SCGE assay for characterizing diminished DNA repair in this setting, we determined the ability of human cancer cell lines harboring mutations in BRCA1 (breast line HCC1937 containing a homozygous deletion and lymphoblastoid line HCC1937L containing a heterozygous deletion) and BRCA2 (pancreatic line Capan-1 containing a homozygous deletion) to repair DNA damage caused by treatment with radiation, etoposide, and hydrogen peroxide. The breast cancer cell line MCF7 and pancreatic cancer cell line BxPC-3 were used as controls. DNA damage was induced in these cell lines by irradiation on ice, 5 minute exposure to hydrogen peroxide, or 1 hour exposure to etoposide. Cells were allowed to repair for 1 hour at 37 deg C and damage measured by the alkaline SCGE. Except for the highest dose of irradiation (10,000 R), where repair was less than 30% for both the MCF7 and HCC1937 lines, repair was proficient for each agent, dose, and cell line. Most cell lines repaired over 90 percent of damage from H2O2, 80 percent of damage from etoposide, and 60 percent of damage from 3,000R, with no consistent differences between BRCA mutant and control lines. Human BRCA deficient cell lines showed proficient repair as measured by the alkaline SCGE assay.

Transient Resistance To High-dose Chemotherapy: Evaluation Of The Use Of DNA-damage Assays To Optimize Treatment Schedule

Richard B. Everson, Susan J. Land, James F. Eliason, Jared L. Klein, and Roy D. Baynes

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ABSTRACT

For drugs that interact with DNA, measures of DNA damage can assess the intracellular availability of active drug at a critical molecular target. Measurements of DNA damage should reflect the integrated effects of all resistance factors, including both recognized mechanisms and unanticipated mechanisms. Thus, molecular monitors of DNA damage could provide an important tool for elucidating the time course of complex changes in resistance factors.

Motivated by a recent clinical trial that demonstrated better survival when the interval between induction chemotherapy and high dose therapy was prolonged, this project is using monitors of DNA damage in patient blood cells to determine whether induction chemotherapy caused transient changes in resistance. We are using the single cell gel electrophoresis (comet) assay and assays for chemical modifications to DNA. Prior to analysis of clinical specimens, studies of the capability of these methods were investigated with MCF7 breast cancer cells and human lymphocytes and bone marrow cells. The cells were incubated at 37°C with various concentrations of 4-OH cyclophosphamide, etoposide, or BCNU.

Each agent produced DNA damage that can be measured in a dose dependent manner by the single cell gel assay. Increases were large: for example, mean levels for damage were 4.5, 18.2, and 166 with 0, 10, and 100 uM BCNU, respectively. At low doses, each agent caused a similar pattern of breakage, while at high doses their pattern of damage could be distinguished. Cisplatin continued the cells, consistent with its ability to cause DNA crosslinks. BCNU caused a pattern suggesting diffusion of relatively constant molecular weight fragments prior to electrophoresis and displacement of these fragments during electrophoresis, a pattern shown to indicate apoptosis. 4-OH cyclophosphamide caused broad diffusion of the DNA, a pattern previously linked with necrosis.

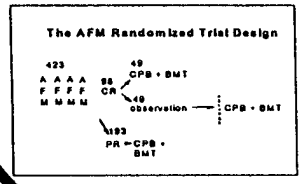
Both lymphocytes and bone marrow cells from patients can be routinely analyzed, allowing study of changes in sensitivity in multiple tissues. It is feasible to use the procedure to study whether induction therapy has a transient effect on resistance to high dose therapy; these studies are underway.

The U.S. Army Medical Research and Material Command under DAMD17-98-1-8352 supported this work.

Introduction

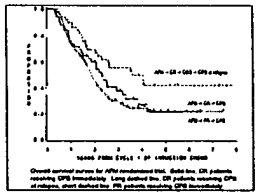
A recent clinical trial showed patients who received high-dose cyclophosphamide, etoposide, BCNU (CPB) therapy at relapse after a complete response to induction therapy had a two-fold greater overall survival than patients who received high dose therapy immediately after induction therapy. The design and results of the trial is presented below and results of the trial are presented in the next column.

The AFM Randomized Trial Design



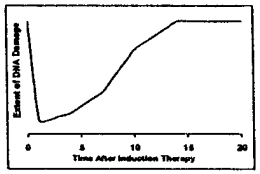
Results of the AFM Randomized Trial

In the AFM Randomized Trial induction therapy was with adriamycin, 5-FU and methotrexate (AFM) and high dose therapy consisted of cyclophosphamide, etoposide, and BCNU (CPB). Immediate treatment was within one month, while treatment after relapse started a median of 21 weeks after relapse. The finding that results were better after immediate treatment runs counter to standard thinking that treatment is likely to be most effective when tumor burden is minimal.



One mechanism that could account for the observation is a transient change in cellular resistance to high dose therapy brought on by induction therapy. As illustrated below, before induction chemotherapy resistance to chemotherapy may be low so that damage induced by a given exposure to high-dose therapy would be high. Induction therapy might then increase resistance, resulting in less DNA damage from the same high-dose therapy. If the induced resistance is transient, levels of DNA damage will increase as time passes after therapy. Thus measurements of levels of damage after therapy could suggest the optimal interval between induction therapy and high-dose therapy - a period where induced damage had returned to a high level but the disease had not been allowed to progress. We developed approaches to test question by analysis of DNA damage.

Hypothetical Changes in DNA Damage after Induction Therapy:



Methods and Results: SCGE/Comet Assays of MCF7 Cells and Human Lymphocytes

The Single Cell Gel Electrophoresis (SCGE or Comet assay) has a number of technical advantages for measuring DNA breakage. It is highly sensitive and can be performed on small numbers of clinical any eukaryotic cell. We developed methods to examine the possibility using the assay to study cultured breast cancer cells, lymphocytes from patients, and immunomagnetic tumor cells isolated with immunomagnetic beads from patient bone marrow.

The SCGE assay is conducted as follows:

- Isolate cells
- Embed cells in agarose
- Lyse cell to remove membrane and proteins leaving DNA
- Denature DNA with high pH
- Electrophorese cells
- Neutralize, dry, and stain (SYBR Green)
- Quantify damage by image analysis

Nucleoids with intact DNA remain circular after electrophoresis. Cells with DNA breakage reveal a brightly fluorescent circular head and a tail of damaged DNA that has electrophoresed faster than the intact DNA. These cells resemble comets, hence the term "comet assay" has been applied widely to the procedure.

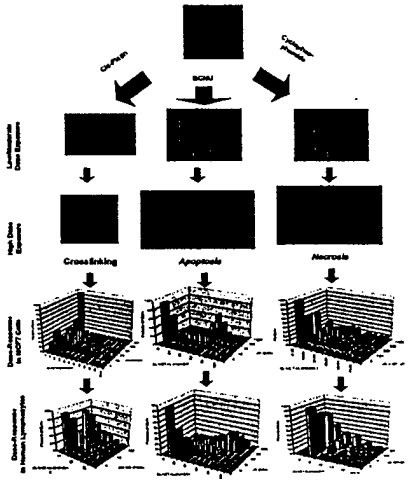
We conducted a series of in vitro experiments establishing dose-response relationships for DNA breakage induced by each of the individual agents used for CBP. The figure at the right summarizes the results of these studies.

Each agent induced DNA breakage at doses comparable to estimated AUCs that would be achievable in vivo with CPB therapy. At low doses the pattern of breakage was greatly similar for each agent. At high doses the pattern of damage differed for each agent. These patterns were consistent for most of the cells assayed. The patterns were also similar to those previously described in the literature for comet damage.

In these studies the pattern of damage caused by high doses differed by for each drug

- Cisplatin constructed the cells, consistent with its ability to cause DNA crosslinks and patterns of Comet damage previously seen with crosslinking agents.
- BCNU caused a pattern suggesting diffusion of relatively constant molecular weight fragments prior to electrophoresis and displacement of these fragments during electrophoresis, a pattern shown to indicate apoptosis in a recent series of studies.
- 4 hydroxy Cyclophosphamide (4-HC) caused broad diffusion of the DNA, a pattern previously linked with necrosis.

Each agent caused damage in a dose-dependent manner in both MCF7 cells and primary lymphocyte cultures, with flattening at very high doses of BCNU and 4OH-CP. There was a reversal at high doses of Cisplatin consistent with its action as a crosslinker. Dose dependent measurements of damage can be made for a broad dose range, avoiding flattening at high doses.



Patterns of Damage and Dose Response Curves for Comet Assays of Agents in CBP.

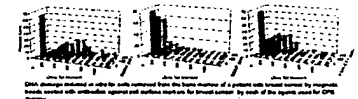
Methods and Results: Analysis of Breast Cancer Cells Separated from Bone Marrow for DNA Breakage

In addition to studying surrogate cells such as lymphocytes, we have developed methods for isolating micrometastatic tumor cells from patient bone marrow and assaying them for DNA damage by the SCGE assay (Lund et al., AACR, 1999). In these studies tumor cells were separated from human bone marrow specimens of breast cancer patients by sequentially incubating the cells with a mixture of 3 mouse anti-human monoclonal antibodies and magnetic beads attached to anti-mouse antibodies (rat anti-mouse IgG (Dynal, Lake Success, NY)). The cells bound to the beads were removed using a magnet. Beads were then prepared with the beads and stained with anti-human cytokeratin antibodies. A cell visualized by magnetic beads is presented below, left. All of the visualized cells stained positive for cytokeratin.

Photomicrograph of tumor cells positive (mets) cancer and marked with beads

Cells Removed from Patient Bone Marrow by Magnetic Beads after in vitro SCGE

Tumor cells were separated from human bone marrow specimens from patients with breast cancer with two or more areas of cytosolic cytokeratin using the 3 mouse anti-human monoclonal antibodies and magnetic beads in the procedure described above. In a typical experiment 1.5 million fresh bone marrow cells yielded approximately 1,400 cells with two or more beads that could be counted in the Comet assay. Controls for BCNU treated cells are illustrated above, and results for 50 control and 50 cells treated for two hours with each of the agents used for CBP are presented below.



DNA damage induced in vitro by cells removed from the bone marrow of a patient with breast cancer by magnetic beads coated with antibodies against and surface markers for breast cancer by each of the agents used for CPB therapy

Conclusions:

- Each of the agents in CPB produces DNA damage that can be measured with the SCGE assays in a dose dependent manner
- At low doses all three agents cause a similar pattern of breakage, while at high doses their pattern of damage could be distinguished
- Both lymphocytes and bone marrow cells from patients can be routinely analyzed, allowing study of changes in sensitivity in multiple tissues
- It is feasible to use the procedure to study whether induction therapy has a transient effect on resistance to high dose therapy

Specimen collection is ongoing to use this approach to study transient resistance to chemotherapy.

The U.S. Army Medical Research and Material Command under DAMD17-98-1-8352 partially supported this work.

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[CANCER RESEARCH 60, 4623–4628, August 15, 2000]

The DNA of Annexin V-binding Apoptotic Cells Is Highly Fragmented[1]

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ABSTRACT

Jurkat leukemia cells induced to undergo apoptosis by treatment with an antibody against the Fas receptor have two annexin V (AV)-binding subpopulations: (*a*) single-positive cells that bind AV but not propidium iodide (PI); and (*b*) double-positive cells that bind AV and PI. The single-positive population is thought to represent an early stage of apoptosis. We have examined the relationship between AV binding and a classical characteristic of apoptosis, DNA fragmentation. Time course studies with Jurkat cells treated for 1, 2, or 4 h with anti-Fas indicated that the proportion of AV-binding cells was increased after 2 h. A significant increase in DNA fragmentation was observed only at 4 h as measured by the mean tail moment determined with the alkaline single cell gel electrophoresis (comet) assay. This correlation suggests a temporal relationship between the two parameters, but does not provide direct evidence of what happens in individual cells. We developed a method to measure fluorescent markers of cellular structure or function with a laser scanning cytometer and then perform the comet assay on the same cells. Cells in each AV-binding subpopulation were re-examined before and after electrophoresis. Most AV^-/PI^- cells had no DNA damage, although a few cells showed a pattern of damage characteristic for apoptosis. Double-positive cells all had damaged DNA; approximately half had the apoptotic pattern, and the rest had a pattern typical for necrosis. Nearly all of the single-positive cells had damaged DNA with the apoptotic pattern. Both AV-positive populations contained cells with little or no detectable DNA after electrophoresis, indicating that the DNA was highly fragmented. These results indicate that AV binding is an excellent marker for apoptotic cells, but that these cells already have fragmented DNA.

INTRODUCTION

Programmed cell death (apoptosis) is an important process in normal development and in tissue homeostasis, as well as a key mechanism by which anticancer therapies exert their cytotoxic effects. Agents like anticancer drugs and ionizing radiation that damage DNA induce apoptosis through a p53-dependent pathway (1). Binding of p53 and other nuclear proteins to the sites of damage in the DNA appears to trigger the apoptotic process. Another mechanism that can induce apoptosis involves the interaction of proteins such as Fas (CD95) or tumor necrosis factor with their receptors on the surface of cells. Signaling from these so-called death receptors starts the apoptotic process (2).

Regardless of how the apoptotic process has been initiated, by intrinsic signals or extrinsic signals involving death receptors, a hallmark of apoptosis is fragmentation of the DNA. Two main steps have been identified for apoptotic DNA fragmentation (3). The first involves formation of high molecular size DNA fragments of 50–300 kb. This process is widely observed in different cells and is propagated through single- and double-strand breaks in the DNA. The second step generates small, 200–300-bp DNA fragments. These small fragments lead to DNA ladder formation classically associated with apoptosis, although it can be absent in some cell types (4).

Another characteristic of cells undergoing apoptosis is the capacity to bind the protein AV.[4] AV binds to PS, which is normally located on the inner leaflet of the plasma membrane, but it is externalized to the outer leaflet during apoptosis. PI staining is widely used to discriminate living cells, which exclude this DNA dye, from dead cells, which are permeable to it. In populations of cells undergoing apoptosis, there are some cells that bind AV but are not stained with PI. This "single-positive" population is thought to represent cells in an early stage of apoptosis because the cells apparently exclude PI and because it appears earlier than DNA ladders can be seen (5).

The Fas-induced apoptotic pathway in Jurkat cells is one of the best-examined models of apoptosis. As in other apoptotic pathways, the activation of cysteine-dependent aspartate-directed proteases, *i.e.*, caspases, is crucial (2). The initial events may be reversible, but in turn, initiate irreversible processes belonging to the "execution phase," such as externalization of phosphatidylserine in the plasma membrane and fragmentation of nuclear DNA.

DNA fragmentation related to apoptosis is usually measured using mass biochemical methods. With heterogeneous cell populations, these techniques will miss contributions from small subpopulations. The comet assay, on the other hand, measures DNA fragmentation on a single cell level, allowing analysis of subpopulations of cells. Our immediate aim was to develop a method to measure the relationship between nuclear DNA fragmentation and membrane PS externalization, two hallmarks of apoptotic cell death on a cell-by-cell basis. We show that there is a high degree of DNA fragmentation in Jurkat cells that stain with AV after apoptosis is induced by treatment with an antibody against Fas.

MATERIALS AND METHODS

Cell Culture. Jurkat T lymphocytic leukemia cells (American Type Culture Collection, Manassas, VA) were kept in continuous logarithmic growth by passaging them at a concentration of 2.5×10^5 cells/ml every other day in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 10 mM HEPES (Sigma, St. Louis, MO), and 50 μg/ml gentamicin (Life Technologies, Inc.).

Induction of Apoptosis. Cells were subcultured 1 day before each experiment was performed. Apoptosis was induced by transfer into fresh culture medium containing 2 μg/ml of a monoclonal antibody against human Fas (CD95; mouse IgG1, clone DX2.1; R&D Systems, Minneapolis, MN) and 2 μg/ml of protein G (Sigma). The cells were incubated at 37°C for various periods of time. In some experiments, control cells were incubated with protein G alone.

AV and PI Staining. After incubation with anti-Fas and or protein G, cells were harvested by centrifugation for 5 min at 200 × g and were resuspended in AB buffer (140 mM NaCl and 2.5 mM $CaCl_2$). Aliquots containing 1×10^5 cells in 100 μl of buffer were stained with 10 μl of PI (50 μg/ml) solution and with 5 μl of FITC-conjugated AV (17.6 μg/ml; PharMingen, San Diego, CA) for 5 min on 37°C. After staining, 400 μl of AB buffer were added to the cells, and samples were stored on ice until data acquisition. Measurements were completed within 1 h.

Flow Cytometry. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Photomultiplier

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[4] The abbreviations used are: AV, annexin V; PI, propidium iodide; PS, phosphatidylserine; LMP, low melting point; LSC, laser scanning cytometer.

voltages were adjusted to have the unlabeled Jurkat cell population fall in the first decade of fluorescence. Cells labeled with only AV-FITC or PI were used to adjust the compensation. Data acquisition and analysis were performed by the CellQuest program (Becton Dickinson).

Single Cell Gel Electrophoresis (Comet Assay). Agarose-coated slides were made by dipping half-frosted slides (Superfrost+, VWR, Batavia, IL) into hot (80°C) 1% agarose (SIGMA) in distilled water and drying in air. A total of 2×10^4 cells were suspended in 80 μl of 0.75% LMP agarose (Boehringer Mannheim, Indianapolis, IN) prepared in AB buffer and layered onto the agarose-coated slides. The agarose was solidified by placing the slides on ice for 2 min. A second layer of 0.75% LMP agarose was applied to cover the cells. The cells were lysed in ice-cold alkaline lysis buffer [1% lauryl-sarcosine, 2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO, 1% Triton-X-100 (pH 10)] for 1 h. Then DNA was unwound for 15 min in cold running buffer (300 mM NaOH, 1 mM EDTA) and electrophoresed for 20 min. Slides were neutralize in cold 0.4 M Tris buffer (pH 7.4) for 5 min and fixed in cold ethanol for 5 min. The slides were stained with SYBR Green I (Molecular Probes, Eugene, OR) diluted 1:1000 in TE [10 mM Tris (pH 8.0) and 2 mM EDTA] for 15 min and covered with antifade solution (ProLong, Molecular Probes). Comets were quantitatively evaluated using a fluorescence microscope (Zeiss, Germany) with the Komet 3.1 image analysis system (Kinetic Imaging, Bromborough, United Kingdom). LMP agarose was prepared in AB buffer.

Tail moment is defined as a product of the distance between the head and tail mass centers and the relative amount of DNA in the tail compared with the total DNA in each comet (6).

Combination Assay Using the LSC. Cells were stained with AV and PI as described above. To each 100-μl aliquot of labeled cells, 300 μl of 1% LMP agarose were added. From this mixture, 80 μl were layered on the surface of each agarose-coated slide. After the agarose with cells solidified, a second layer of agarose was added, and coverslips were place on top. The slides were kept on ice until measurements were done using the LSC (CompuCyte Corporation, Cambridge, MA). The hardware was controlled by the WinCyte 3.1 software for Windows NT. The 488-nm argon-ion laser line was used for excitation. Green fluorescence and red fluorescence emission as well as forward scatter light was collected. The contour threshold was setup using the "added" parameter, which is a sum of the forward scatter light, green fluorescence, and red fluorescence parameters. Compensation was determined using single-labeled samples. We have used a 10× objective to reduce the time needed for scanning a given area. The size of an apoptotic comet is in the range of 100 μm, which determines maximal cell concentration that can be used. Under our experimental conditions, 3000 cells on each slide could be scanned in ~10 min. After the AV-PI measurements were completed, coverslips were removed from the slides, and they were immersed in the lysis buffer. The DNA was stained with SYBR Green I, neutralized, and fixed as described above. To perform the comet assay, the cells of interest were relocated by the LSC based on the previous measurements. The video signal from the color video camera of the LSC was ported over to the Komet 3.1 image system.

RESULTS

The LSC provides quantitative fluorescence data similar to that obtained by flow cytometry. With Jurkat cells treated with anti-Fas and stained with AV-FITC and PI, both methods identify three distinct populations (*e.g.*, see Fig. 3*A*): double-negative cells (AV^-/PI^-), single-positive cells (AV^+/PI^-), and double-positive cells (AV^+/PI^+). Fig. 1*A* summarizes the results of several of experiments examining the time course of appearance of AV^+ single-positive cells after treatment with anti-Fas. Both cytometric measurements show a similar pattern, although at each time point, the LSC detects more AV^+ cells than does the flow cytometer. Because the LSC is a microscope-based measurement and the cells remain attached to the slide, each event can be re-examined and each could be confirmed to be a cell (see Fig. 3). One h after induction, there was only a small increase in the percentage of single-positive cells, but by the second hour, ~15% of the cells measured were positive by the LSC and 5% by flow cytometry.

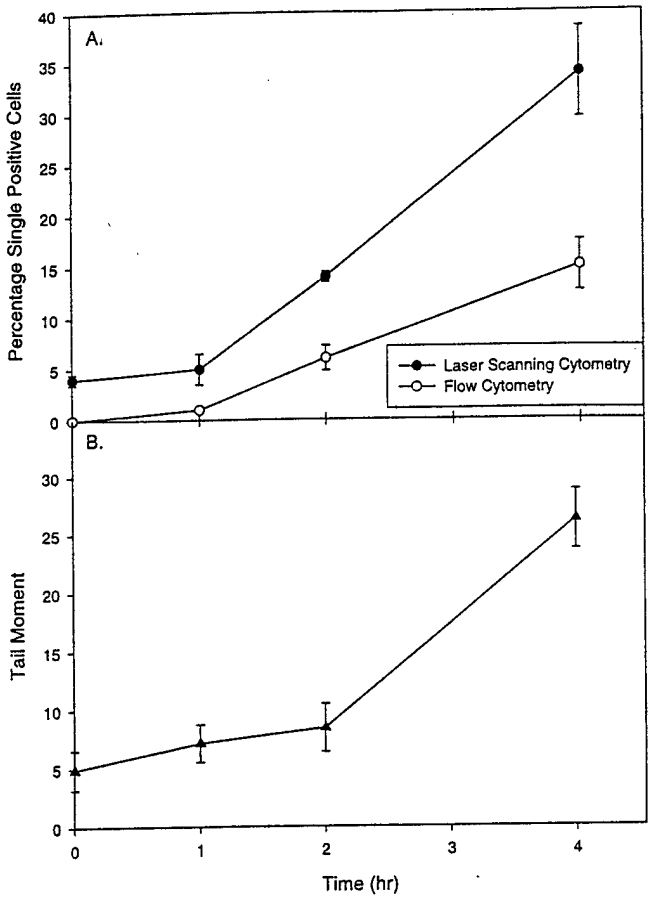

Fig. 1. Time course of induced apoptosis in Jurkat cells measured by flow cytometry, laser scanning cytometry, or the comet assay. *A*, ○, mean percentage of AV^+/PI^- cells from three independent experiments using the flow cytometer. ●, mean percentage of AV single-positive cells from 12 (0 h), 6 (1 h), 8 (2 h), or 4 (4 h) independent experiments using the LSC. *B*, ▲, mean tail moments measured for 100 cells each from five independent experiments. *Vertical bars*, ±1 SE.

The kinetics of apoptosis in these cells were also examined using the standard comet assay. After electrophoresis and staining, 100 cells were randomly selected and scored for DNA damage as measured by the tail moment. The results (Fig. 1*B*) indicate a slow increase in the mean tail moment during the first 2 h, with a large increase by 4 h after induction. More detailed results for the 0- and 2-h time points for a typical experiment are shown in Fig. 2. The histograms for tail moments are depicted in Fig. 2, *A* and *B*. The relationships between tail moments and total DNA staining are shown in Fig. 2, *C* and *D*. The tail moments for control cells were very low, with most being ≤10 (Fig. 2*A*). After 2 h of treatment with anti-Fas, some tail moments were considerably >40.

The time course studies depicted in Fig. 1 suggest that the increase in tail moments of the anti-Fas-treated cells may occur somewhat later than the increase in AV binding. However, this approach does not provide information as to how much DNA fragmentation is present in each AV^+ cell. To examine this more precisely, analyses were performed using the LSC. Cells in each area of interest of the scattergrams were relocated. Representative bright field and epifluorescence photomicrographs were taken of these cells. They were then lysed and electrophoresed for the comet assay. The locations of the cells of interest were re-examined to determine the extent of DNA fragmentation.

The results of a representative experiment are shown in Fig. 3. The bright field photograph of a typical double-negative cell shows a

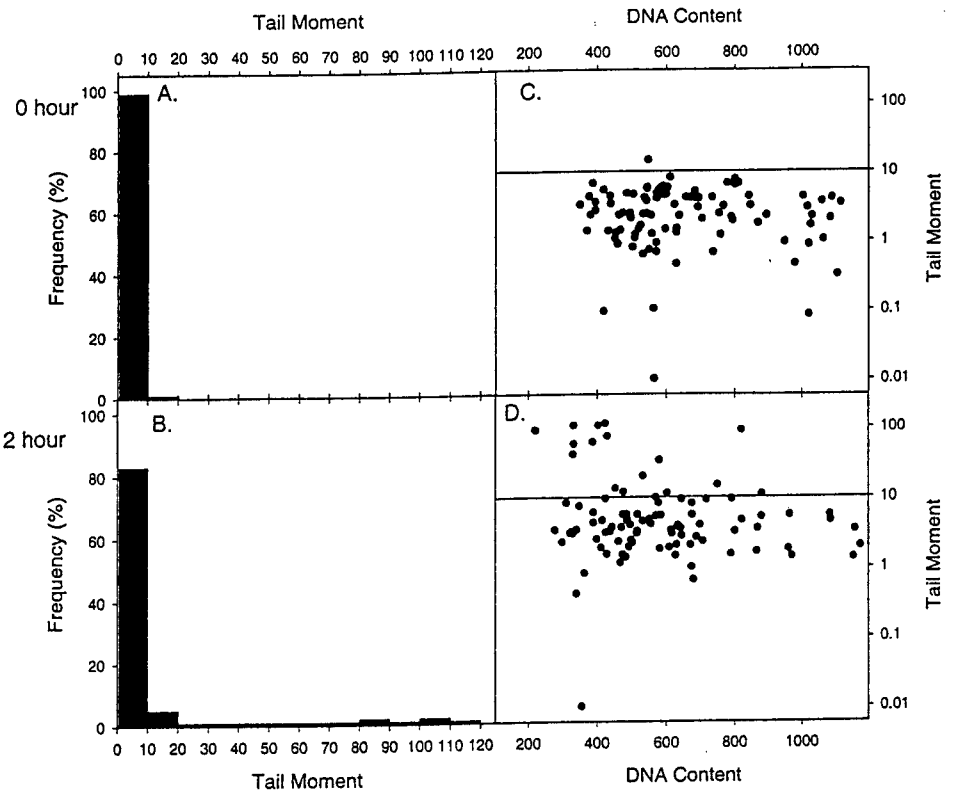

Fig. 2. Distribution of tail moments (*A* and *B*) and relationships to DNA content (*C* and *D*) in Jurkat cells without (*A* and *C*) and with (*B* and *D*) anti-Fas for 2 h. The results for a representative experiment from a series of seven are depicted.

round cell with a sharply defined cell membrane (Fig. 3*B1*). After electrophoresis, the nuclei of most cells in this population remained as bright circles (Fig. 3*B2*). The cells in the double-positive population had indistinct borders when viewed under a bright field (Fig. 3*C1*). AV staining on these cells was usually uniform as shown in Fig. 3*C2*. Many of the cells in this population gave a typical "comet" pattern for their DNA after electrophoresis (Fig. 3*C3*) with a bright "head" where the nucleus was and a tail of damaged DNA. This pattern is characteristic of necrotic cells The single-positive cells had some definition around them, but their shapes were much more irregular than those of the double-negative population (Fig. 3, *D1–3*) and often showed localized staining with AV around the edges (Fig. 3, *D4–6*). The DNA patterns are typical of apoptotic cells (Fig. 3, *D6–9*), forming clouds of small pieces of DNA that migrate away from the original site, some with only traces of DNA staining (Fig. 3*D8*).

Based on analysis of a number of experiments, the DNA patterns of PI/AV-stained cells could be placed in four basic categories: (*a*) normal nuclei without DNA damage having all DNA remaining at the site of the nucleus (Fig. 3*B2*); (*b*) "Comet" with considerable DNA both in the head and in the tail, characteristic of necrotic DNA damage (Fig. 3*C3*); (*c*) "Apoptotic comets" with little DNA in the head, usually as a ring with less DNA in the center of the head, followed by

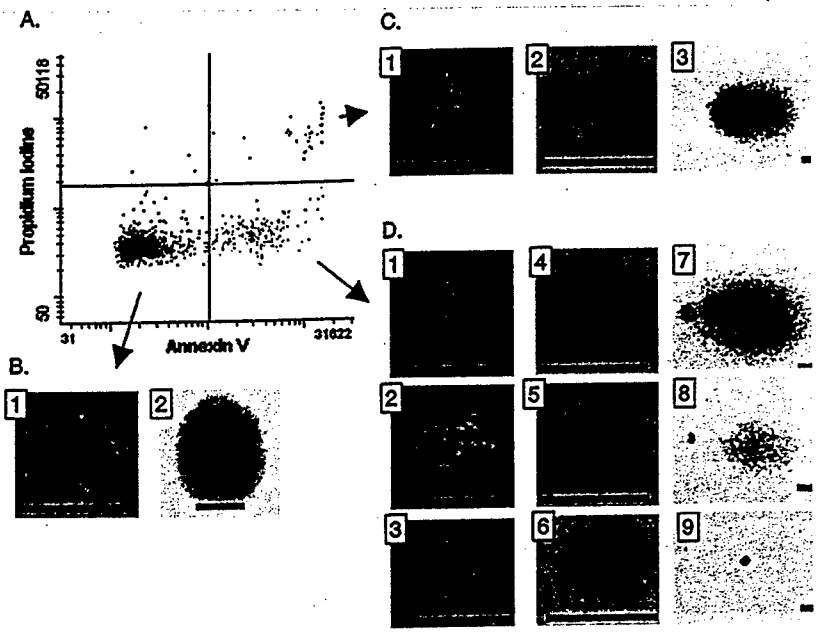

Fig. 3. Typical bright field and fluorescence images of Jurkat cells treated for 2 h with anti-Fas in different AV-FITC- and PI-staining subpopulations. After staining, the cells were embedded in agarose on a slide, and fluorescence was measured using the LSC. Events were relocated, and images were made for each subpopulation identified in the scatter plot (*A*). Bright field images of cells (*B1, C1, D1, D2,* and *D3*) as well as AV fluorescence images (*C2, D4, D5,* and *D6*) were recorded before lysis and electrophoresis, after which SYBR Green I fluorescence images were recorded (*B2, C3, D7, D8,* and *D9*). The characteristic DNA staining patterns after conducting the comet assay are the following: normal DNA (*B2*), "necrotic" comet (*C3*), "apoptotic" comet with measurable DNA (*D7*), "apoptotic remnant" with trace DNA in its tail (*D8*), and "apoptotic remnant" without DNA (*D9*). Each row shows a set of images obtained sequentially from the same cell. *Bars,* 10 μM. As can be seen, the images of the comets were taken with a lower magnification, and the stage was moved to center the comet because the LSC centers on the original location of the cells, where the apoptotic remnants are found.

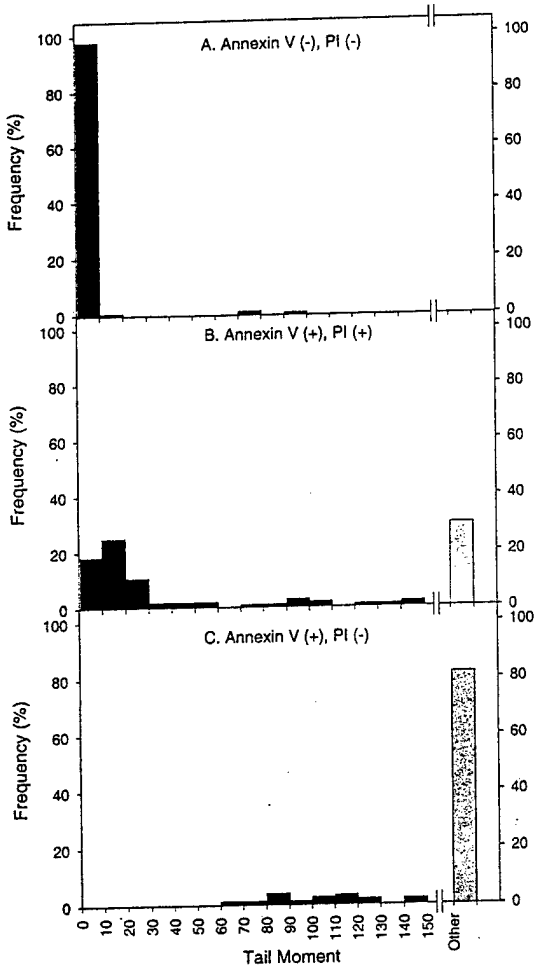

Fig. 4. Distributions of tail moments in AV^-/PI^- (*A*), AV^+/PI^+ (*B*), and AV^+/PI^- (*C*) Jurkat cells after treatment with anti-Fas for 2 h. "Other" consists of "apoptotic remnants" and those "apoptotic" comets where it was not possible to measure tail moments because there was little or no DNA remaining at the cellular locations after electrophoresis. The results are for a representative experiment from a series of six.

a large cloud. This is characteristic of apoptotic DNA fragmentation (Fig. 3*D7*). In certain cases, at the sites of nuclei, only traces of DNA remained, appearing as ring-shaped objects (not shown). Classification of these based on the relocalization of a large number of cells and the presence of DNA clouds with various fluorescent intensities indicates that they represent a continuum with progressively less DNA; and (*d*) Small condensed particles at the original locations of the nuclei with small amounts of fragmented DNA in a cloud (see left side of Fig. 3*D8*) or even without a DNA tail (Fig. 3*D9*). The color of these condensed particles on SYBR Green I-stained slides was more yellow than the stained DNA. Because SYBR Green I can also stain hydrophobic proteins, this may be an indication that they were not composed of pure DNA. We will refer to these as "apoptotic remnants."

These patterns are segregated between the different subpopulations in the AV-PI scattergrams. Table 1 shows the proportions of each in these populations of cells treated with anti-Fas for 2 h and control cells. The double-negative population primarily consisted of cells with unfragmented DNA. In both treatment groups, >90% of the cells in this population had intact DNA. The AV single-positive population in either treatment group demonstrated highly fragmented apoptotic DNA and apoptotic remnants, with no cells having a necrotic pattern and only a few with undamaged DNA. In the double-positive population, there were both necrotic and apoptotic comets. Although treatment changes the numbers of cells in the single- and double-positive subpopulations compared with untreated cells, the type of damage in each subpopulation is the same.

We have measured the tail moments associated with each subpopulation of cells by transferring the signal from the video camera on the LSC to the image analysis system for the comet assay. Many apoptotic events in the single-positive and double-positive populations could not be scored because the total amount of DNA was too low for accurate image analysis. This includes some of the apoptotic comets that had lost most of their DNA as well as the apoptotic remnants. These were counted manually and would not have been identified in the standard alkaline comet assay. The results are shown in Fig. 4 and Table 1. The double-negative population had unfragmented nuclei, with 96.6% of the cells having tail moments <10 (Fig. 4*A*). After anti-Fas treatment, the proportion of cells with normal nuclei dropped to 90.8%, and those with apoptotic patterns increased from 3.0 to 8.6% (Table 1). In the double-positive population, there was a wide range of values with necrotic comets having tail moments between 10 and 40 and apoptotic comets with tail moments >40 (Fig. 4*B*; Table 1). This population also contained apoptotic remnants. Most of the events in the single-positive populations could not be measured, and those that could had tail moments >60 (Fig. 4*C*). All represented apoptotic patterns. In cells treated with anti-Fas, there was a shift to earlier stages of apoptosis, with apoptotic comets increasing from 35 to 85% and apoptotic remnants decreasing from 58 to 12% of the population. Thus, as cells are induced into apoptosis, earlier stages predominate (Table 1).

The relative proportions of measurable apoptotic comets to late apoptotic events, such as apoptotic remnants that could not be characterized by tail moment measurements in the single-positive population of cells treated with anti-Fas, were calculated. After untreated cells have been harvested and prepared for measurement (0 h), ~5% are single positive (Fig. 1), and 85% of these do not have sufficient DNA to measure comet tail moments. As the number of single positive cells increases after the addition of anti-Fas, the proportion of measurable comets increases progressively from 15 to 22%, and then to 29%, after 1 and 2 h. By the third hour of treatment, 38% of the comets represent these early stages as new cells are induced to undergo apoptosis. This proportion falls rapidly again to 12% in the next hour.

Similar studies were performed with MCF-7 breast cancer cells that had been serum-starved to induce apoptosis and with PC3 prostate cancer cells treated with ionizing radiation. The results showed that the AV single- and double-positive populations had similar DNA

Table 1 *Pattern of DNA damage in cells labeled with AV and PI after 2-h treatment with anti-Fas*[a]

Pattern:	Normal DNA (%)		Necrotic comet (%)		Apoptotic comet (%)		Apoptotic remnant (%)	
Anti-Fas:	−	+	−	+	−	+	−	+
AV^-/PI^-	96.6 ± 1.1	90.8 ± 3.2	0.4 ± 0.4	0.6 ± 0.4	2.2 ± 0.8	7.2 ± 2.2	0.8 ± 0.6	1.4 ± 1.2
AV^+/PI^-	6.5 ± 1.2	2.4 ± 1.3	0 ± 0	0 ± 0	35.0 ± 2.0	85.3 ± 5.8	58.5 ± 4.7	12.3 ± 4.5
AV^+/PI^+	0 ± 0	0 ± 0	56.2 ± 5.5	45.1 ± 1.4	23.9 ± 1.5	38.7 ± 2.5	19.9 ± 1.1	16.2 ± 0.7

[a] Results represent averages from four experiments ± 1 SE. At least 100 cells were counted in each experiment for each AV, PI subpopulation, when possible. Data are expressed as percentages.

patterns to those of Jurkat cells induced with anti-Fas. Single-positive cells were all apoptotic, with a high degree of fragmentation and DNA loss appearing the same as those in Fig. 3*D*. Double-positive cells, on the other hand, had a mixture of apoptotic and necrotic comets similar to those in Fig. 3*C*.

DISCUSSION

The process of apoptosis has received a great deal of attention in experimental oncology. Defects in this pathway early during carcinogenesis can ultimately lead to cancer formation (7) and later to resistance to cancer treatment (1, 8). Several pathways have been described for apoptosis in different systems, but they all converge with activation of the protease caspase 3 and subsequently of endonuclease activity that results in fragmentation of nuclear DNA (4, 9–13). Experimentally, this DNA fragmentation has been demonstrated in model systems as a "ladder" following electrophoretic separation of DNA extracts prepared from the cell population. This method lacks sensitivity because the proportion of apoptotic cells in the population must be large to be detected. A method has been developed to detect DNA damage in single cells called the "comet" or single cell gel electrophoresis assay that can detect effects in subpopulations of cells (14).

The binding of AV by cells undergoing apoptosis has been proposed to be an early event based on correlative studies on cell populations (5): (*a*) increases in the single AV^+ cell population occur much earlier than increases in the double-positive cell population; (*b*) the proportion of AV-binding cells is higher at all times after induction than that of cells with morphological changes in the nucleus; (*c*) AV binding appeared earlier than cell shrinkage as determined by flow cytometry. Until recently, studies of this type have been correlative by necessity because assays such as AV binding, which require viable cells, and morphological assays could not be performed on the same cell (15). We have developed a method using the ability of the microscope-based LSC to relocate cells that combines the AV-binding assay with assessment of DNA fragmentation by the comet assay in the same cells.

To validate our approach, we selected a well-studied apoptotic system, Jurkat leukemia cells induced to undergo apoptosis by an antibody against Fas. In contrast to other model systems in which drug- or radiation-induced DNA damage initiates the apoptotic program, anti-Fas interacts with a cell surface death receptor to initiate the process. To ensure that we were examining the early events of apoptosis, we have used treatment times of no more than 4 h.

Our initial studies comparing the results obtained by flow cytometry and laser scanning cytometry for PI-AV-FITC-stained cells demonstrated that there was no significant difference between the two methods in the time course of appearance of single- and double-positive cell populations, confirming the results of Bedner *et al.* (11). However, the proportions of AV^+ cells measured by laser scanning cytometry were higher than those determined by flow cytometry. There are two possible explanations for this difference. The first is that the embedding of the cells in agarose immediately after staining preserves fragile apoptotic cells that may be lost in flow cytometry because of pressure changes through the flow aperture. The second possibility is that the cells might remain intact during flow analysis, but that changes during the apoptotic process in the side scatter parameter used to set regions for flow analysis might remove these cells from the region being analyzed (16). In either case, each positive event detected with the LSC could be confirmed visually as a cell. Another advantage of embedding the cells in agarose is that all of the cells can be examined, avoiding the potential problem associated with other microscopic assays in which late apoptotic cells may be lost because they cannot attach to glass surfaces (11).

After lysis, electrophoresis, and ethanol fixation, it was possible to reliably relocate nuclei at the sites where cells were first detected for the PI and AV measurements. There was no PI or FITC fluorescence remaining at these locations. When objects were relocated, it was nearly always possible to classify them as intact nuclei, necrotic comets, apoptotic comets, or apoptotic remnants. At the location of single- or double-positive cells, damaged DNA with either an apoptotic or necrotic pattern was evident or small-condensed particles were found. The size of these irregularly shaped particles was ~2–4 μm in diameter, and their color was more yellow than that of normal DNA stained with SYBR Green I. They were also visible on bright field images in contrast to apoptotic or necrotic DNA. These findings suggest that apoptotic remnants are composed of heavily condensed material, probably cross-linked proteins. It has been shown that transglutaminases are activated during the last stages of apoptosis, resulting in cross-linked cellular proteins that prevent disintegration of the apoptotic cells (17).

We did not observe comets with intermediate levels of DNA damage in the double-negative or single-positive populations in these experiments. Most double-negative cells had undamaged DNA with tail moments <10, whereas the single-positive population was composed primarily of heavily damaged cells with apoptotic fragmentation patterns having tail moments >40. In some apoptotic cells, tail moment was not possible to calculate because little or no DNA remained in the head. These observations are in good agreement with field inversion gel electrophoresis data, where an abrupt (within 15 min) DNA fragmentation was found in apoptotic cells (18). We observed intermediate DNA damage (tail moments between 10 and 40) only in the double-positive population, and these cells had comet shapes typical of necrotic DNA damage (19). Thus, it appears that in apoptotic cells, the transition from undamaged DNA to a high degree of DNA fragmentation is very rapid. After the initial fragmentation that gives rise to measurable comets, the DNA is further fragmented, giving rise to comets in which all of the DNA has migrated from the nuclear area during electrophoresis. Finally, there is what appears to be a gradual loss of DNA from the comets leaving only the apoptotic remnants. This last stage may take between 6 and 12 h in Jurkat cells induced by anti-Fas (5).

When measured with the LSC, there was a background of apoptotic cells in the control samples (0 h). These cells that underwent spontaneous apoptosis were all in the late stages of apoptosis. When cells were induced to undergo apoptosis with anti-Fas, the numbers of cells in the early stages increased gradually through the first 3 h and then decreased. This suggests that an initial wave of apoptosis occurs during the first 3 h. Whether or not further waves occur in this population and their relationships to proliferative status of the cells at the time of treatment remain to be determined. Our results confirm that AV binding is an excellent marker for apoptotic cells (5, 20, 21). Virtually all of the cells in the single-positive population had apoptotic comets or apoptotic remnants. In contrast, the double-positive population was composed of cells giving rise to comets having necrotic or apoptotic patterns in about equal numbers. This means that counting only the single-positive cells will underestimate the actual numbers of apoptotic cells.

We have further shown that AV binding does not precede DNA fragmentation because all of the single-positive cells, which are purported to be earlier in the apoptotic process than double-positive cells, have highly fragmented DNA. In contrast to our data, it has been suggested that AV binding is an earlier step in the execution phase than DNA fragmentation (21, 22), based on use of the TUNEL assay to measure DNA fragmentation. This method is not sensitive enough

to detect high molecular size DNA fragmentation that occurs in the early phases of apoptosis, but only detects the final internucleosomal DNA degradation (23, 24). The comet assay, on the other hand, also detects the 50-kb DNA fragments in apoptotic cells (14), so we are detecting the earlier high molecular size fragmentation in our assays using the comet assay. Furthermore, we are looking at early times after the addition of anti-Fas, and if PS exposure was an earlier event than DNA fragmentation, we would expect to find cells containing undamaged DNA in the single- or double-positive populations at these times, which is not the case. Our findings that AV-binding cells have a high degree of DNA fragmentation and loss are also supported by the results of Martin *et al.* (5) showing a correlation between the proportions of peripheral blood neutrophils binding AV after anti-Fas or drug treatment and the numbers of nuclei with sub-G_0/G_1 DNA content as determined by flow cytometry. Our studies with MCF-7 and PC3 demonstrate that the relationship between AV binding and DNA fragmentation is not restricted to cells of hematopoietic origin.

In conclusion, our method using the LSC to relocate cells after subpopulations have been identified and to measure DNA damage by the comet assay provides a powerful tool for studies on apoptosis. This technique can be used to examine other markers of apoptosis, such as binding of mitochondrial specific dyes, to determine their relationship to DNA damage in apoptotic cells. It will also allow analysis of biological relationships between virtually any cell surface or cytoplasmic marker and DNA damage in individual cells.

REFERENCES

1. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (Lond.), *362:* 847–849, 1993.
2. Nagata, S. Apoptosis by death factor. Cell, *88:* 355–365, 1997.
3. Earnshaw, W. C. Nuclear changes in apoptosis. Curr. Opin. Cell Biol., *7:* 337–343, 1995.
4. Khodarev, N. N., Sokolova, I. A., and Vaughan, A. T. Mechanisms of induction of apoptotic DNA fragmentation. Int. J. Radiat. Biol., *73:* 455–467, 1998.
5. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of *Bcl-2* and *Abl*. J. Exp. Med., *182:* 1545–1556, 1995.
6. Olive, P. L., Banath, J. P., and Durand, R. E. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat. Res., *122:* 86–94, 1990.
7. Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. Tumor spectrum analysis in p53-mutant mice. Curr. Biol., *4:* 1–7, 1994.
8. Moos, P. J., and Fitzpatrick, F. A. Taxane-mediated gene induction is independent of microtubule stabilization: induction of transcription regulators and enzymes that modulate inflammation and apoptosis. Proc. Natl. Acad. Sci. USA, *95:* 3896–3901, 1998.
9. Khodarev, N. N., Sokolova, I. A., and Vaughan, A. T. Association between DNA cleavage during apoptosis and regions of chromatin replication. J. Cell. Biochem., *70:* 604–615, 1998.
10. Inohara, N., Koseki, T., Chen, S., Wu, X., and Nunez, G. CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor. EMBO J., *17:* 2526–2533, 1998.
11. Bedner, E., Li, X., Gorczyca, W., Melamed, M. R., and Darzynkiewicz, Z. Analysis of apoptosis by laser scanning cytometry. Cytometry, *35:* 181–195, 1999.
12. Liu, X., Zou, H., Slaughter, C., and Wang, X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell, *89:* 175–184, 1997.
13. Tang, D., and Kidd, V. J. Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis. J. Biol. Chem., *273:* 28549–28552, 1998.
14. Olive, P. L., and Banath, J. P. Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA cross-linking agent. Exp. Cell Res., *221:* 19–26, 1995.
15. Li, X., and Darzynkiewicz, Z. The Schrodinger's cat quandary in cell biology: integration of live cell functional assays with measurements of fixed cells in analysis of apoptosis. Exp. Cell Res., *249:* 404–412, 1999.
16. Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., and Traganos, F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry, *27:* 1–20, 1997.
17. Fesus, L. Transglutaminase-catalyzed protein cross-linking in the molecular program of apoptosis and its relationship to neuronal processes. Cell. Mol. Neurobiol., *18:* 683–694, 1998.
18. Weis, M., Schlegel, J., Kass, G. E., Holmstrom, T. H., Peters, I., Eriksson, J., Orrenius, S., and Chow, S. C. Cellular events in Fas/APO-1-mediated apoptosis in JURKAT T lymphocytes. Exp. Cell Res., *219:* 699–708, 1995.
19. Fairbairn, D. W., Walburger, D. K., Fairbairn, J. J., and O'Neill, K. L. Key morphologic changes and DNA strand breaks in human lymphoid cells: discriminating apoptosis from necrosis. Scanning, *18:* 407–416, 1996.
20. van Engeland, M., Kuijpers, H. J., Ramaekers, F. C., Reutelingsperger, C. P., and Schutte, B. Plasma membrane alterations and cytoskeletal changes in apoptosis. Exp. Cell Res., *235:* 421–430, 1997.
21. van Engeland, M., Nieland, L. J., Ramaekers, F. C., Schutte, B., and Reutelingsperger, C. P. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry, *31:* 1–9, 1998.
22. O'Brien, I. E., Reutelingsperger, C. P., and Holdaway, K. M. Annexin-V and TUNEL use in monitoring the progression of apoptosis in plants. Cytometry, *29:* 28–33, 1997.
23. Godard, T., Deslandes, E., Lebailly, P., Vigreux, C., Sichel, F., Poul, J. M., and Gauduchon, P. Early detection of staurosporine-induced apoptosis by comet and annexin V assays. Histochem. Cell Biol., *112:* 155–161, 1999.
24. Gorczyca, W., Bigman, K., Mittelman, A., Ahmed, T., Gong, J., Melamed, M. R., and Darzynkiewicz, Z. Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. Leukemia, *7:* 659–670, 1993.